From cell surface to nucleus: Unraveling cancer metastasis and the role of nucleophosmin in breast cancer

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ABSTRACT

The most deadly aspect of cancer is its ability to spread from its original location to other sites in the body and grow as distant metastases. Formation of metastases is a multistep process and metastases can form even decades after the removal of the primary tumor. Cell surface proteins are known to play central roles in the adhesive contacts and molecular interactions between the tumor cell and the stroma during various stages of metastasis. In addition, they mediate important signals to intracellular proteins. As the detailed mechanisms of metastasis are still unclear, the aim of this thesis was to discover novel metastasis-associated cell surface proteins for further investigation.

This thesis established an optimized method for the isolation of biotinylated cell surface proteins for proteomic identification. This method was applied to compare the cell surface proteins isolated from an isogenic pair of human MDA-MB-435 cancer cell line with opposite metastatic phenotypes. We found 29 differentially expressed proteins and analyzed the molecular pathways they were involved in. Of these proteins expression of CD109 was shown to mark metastatic melanoma cells and invasive breast cancer cells.

Nucleophosmin (NPM) is a multitasking protein with both oncogenic and tumor suppressive functions. In our comparative proteomics analysis we discovered NPM to be expressed on the surface of the non-metastatic subclone of the MDA-MB-435 cells. We showed that NPM was detected at different localizations in the non-metastatic and metastatic cells most likely due to the expression of novel NPM splice variants discovered in this thesis work. In addition, we showed that expression level of NPM is one mechanism affecting its localization. In regards to patient prognosis, we revealed that high levels of NPM were expressed in the luminal epithelial cells of histologically normal breast tissue and that high levels independently associated with good prognosis in the luminal A breast cancer subtype. On the contrary, novel granular staining pattern and Threonine199 phosphorylation of NPM (NPMpThr199) correlated with aggressive characteristics, basal subtype and poor prognosis of human breast cancer. Moreover, NPMpThr199 associated with expression of a recently identified oncogene, cancerous inhibitor of protein phosphatase 2 (CIPA2).

In brief, this study provides several novel metastasis associated cell surface proteins for future investigation. By using breast cancer tumor microarrays from two large breast cancer patient cohorts and cellular models this thesis demonstrates for the first time, that different NPM forms play divergent and opposite roles in breast cancer.

TIIVISTELMÄ

Syöpä on sairaus, jossa kehon solut jakaantuvat kontrolloimattomasti ja leviävät alkuperäisestä kasvupaikastaan elimistön muihin kudoksiin muodostaen niihin etäpesäkkeitä. Tämä johtaa potilaan kuolemaan. Syövän leviäminen ja etäpesäkkeiden muodostuminen on monivaiheinen prosessi, jonka kaikkia yksityiskohtia ei vielä tarkkaan tunneta. Koska syöpäsolut hyödyntävät solujen pinnalla ilmentyneitä proteiineja kommunikoidessaan ympäristönsä kanssa, näillä proteiineilla on tärkeä rooli syövän leviämisessä uusin kasvupaikkoihin. Väitöskirjatyöni tarkoituksena oli tunnistaa etäpesäkkeiden muodostumisen kannalta tärkeitä solun pintaproteiineja, joita ei ole aikaisemmin yhdistetty syövän leviämiseen.

Tutkimuksessa löysimme 29 proteiinia, joiden ilmentymisellä syöpäsolujen pinnalla oli yhteys solujen kykyyn muodostaa etäpesäkkeitä. Yksi löytämistämme proteiineista oli CD109, jota löytyi sekä etäpesäkkeistä eristetyistä melanoomasoluista että rintasyöpäsoluista, jotka pystyivät leviämään ympäröivään kudokseen. Jatkotutkimuksissa tulisi selvittää, voisiko CD109:n ilmentymistä käyttää syövän ennusteellisena tekijänä. Lisäksi tulisi selvittää, miten CD109:n läsnäolo syöpäsolujen pinnalla auttaa niitä leviämään elimistössä.

Tutkimuksen toisessa osassa löysimme merkittävän yhteyden nukleofosmiini-proteiinin (NPM) ilmentymistason ja potilaiden ennusteen välillä laajassa rintasyöpäaineistossa. Potilailla, joiden syöpäkasvaimissa NPM:n määrä oli vähentynyt, oli suurentunut riski etäpesäkkeiden muodostumiseen ja rintasyövästä aiheutuvaan kuolemaan. Lisäksi osoitimme solukokeissa NPM:n vähentävän rintasyöpäsolujen aggressiivisuutta. Nämä tuloksemme tukevat NPM:n tuumorisuppressiivista (kasvua estävää) toimintaa rintasyövässä. Toisaalta havaitsimme, että potilaiden huonoon ennusteeseen ja etäpesäkkeiden muodostumiseen vaikuttaa NPM:n treoniini-199-fosforylaatio. Havainto on merkittävä, sillä NPM:n toiminta on usein häiriintynyt kasvaimissa, mutta tämän häiriön taustalla vaikuttavia mekanismeja ei tarkkaan tunneta. Tutkimustuloksemme osoittavat sekä NPM:n ilmentymismäärän että sen fosforylaation vaikuttavan tämän proteiinin toimintaan rintasyövässä.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals. Unpublished material is also presented.

I. Piia-Riitta Karhemo, Suvi Ravela, Marko Laakso, Ilja Ritamo, Olga Tatti, Selina Mäkinen, Steve Goodison, Ulf-Håkan Stenman, Erkki Hölttä, Sampsa Hautaniemi, Leena Valmu, Kaisa Lehti, Pirjo Laakkonen. An Optimized Isolation Of Biotinylated Cell Surface Proteins Reveals Novel Players In Cancer Metastasis, J Proteomics. 2012 Dec 21;77:87-100.
doi: 10.1016/j.jprot.2012.07.009

II. Piia-Riitta Karhemo, Antti Rivinoja, Johan Lundin, Maija Hyvönen, Anastasiya Chernenko, Johanna Lammi, Harri Sihto, Mikael Lundin, Päivi Heikkilä, Heikki Joensuu, Petri Bono, Pirjo Laakkonen. An Extensive Tumor Array Analysis Supports Tumor Suppressive Role for Nucleophosmin in Breast Cancer, Am J Pathol. 2011 August; 179(2): 1004–1014. doi: 10.1016/j.ajpath.2011.04.009

III. Piia-Riitta Karhemo, Harri Sihto, Anni Laine, Antti Rivinoja, Petri Bono, Henna Moore, Päivi Rajahalme, Marikki Laiho, Jukka Westermarck, Heikki Joensuu and Pirjo Laakkonen. Novel players in oncogenity of NPM? (manuscript)

ABBREVIATIONS

AML	acute myelod leukemia
ARF	p14ARF
ATP	Adenosine-5'-triphosphate
BLBC	basal like breast cancer
CIP2A	cancerous inhibitor of protein phosphatase 2
CD109	cluster of differentiation 109
CDK	cyclin-dependent kinase
CK2	casein kinase 2
CK5/6	cytokeratin-5/6
CSCs	cancer stem cells
CTCs	circulating tumor cells
DTCs	disseminated tumor cells
ECM	extracellular matrix
ECGFP	enhanced cyan green fluorescent protein
EGFR	epidermal growth factor receptor
EMT	epithelial to mesenchymal transition
ER	estrogen receptor
GRK5	G protein-coupled receptor kinase 5
HER1	human epidermal growth factor receptor-1
HER2	human epidermal growth factor receptor-2
IGSF8	immunoglobulin superfamily member 8
ITGA6	integrin-α6
ITGB1	Integrin-β1
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
MDM2	murine double minute 2
MEFs	mouse embryonic fibroblasts
NPM	nucleophosmin
pThr199	threonine 199 phosphorylation
PP1	protein phosphatase 1

PR	progesterone receptor
PROCR	endothelian protein C receptor
PTGFRN	prostaglandin receptor negative regulator
PTP	protein tyrosine phosphatase
PTPRF	receptor-type tyrosine-protein phosphatase F
RB	retinoblastoma protein
rRNA	ribosomal RNA
SENP	SUMO1/sentrin/SMT3 specific peptidase
SSR4	Translocon associated protein delta subunit
SUMO	Small ubiquitin-like modifier
TICs	tumor initiating cells
TMA	tissue microarray
TME	tumor microenvironment
TNBC	triple negative breast cancer
VAMPA	membrane protein-associated protein A
2D	two dimensional
3D	three dimensional

1 INTRODUCTION

Cancer comprises a large group of diseases caused by deregulated cell growth and resistance to cell death in any part of the body. Every fourth person in Finland is diagnosed with cancer at some point of their life (Cancer Statistics at Finnish Cancer Registry) and according to the World Health Organizations GLOBOCAN 2008 project 7.6 million annual cancer deaths are reported worldwide (Ferlay et al., 2011). The most deadly aspect of cancer is its ability to spread from its original location to other sites in the body in a process referred to as metastasis. Metastasis is a multistep process, details of which are not yet fully understood. Therefore, there is a need to identify novel proteins and molecular pathways that are involved in the regulation of cancer metastasis to better understand and disrupt the process.

Proteins perform many important cellular functions. Mutations in protein encoding genes can modify protein function in many diseases such as cancer (Futreal, 2004). To understand how cancer cell acquires a metastatic phenotype, it is important to know the quantities of different proteins in normal and malignant cells and understand how these quantities change. Proteomics techniques are used to study proteins present in a cell, tissue or an organism at given time and can be applied to find changes in proteins and their quantities between different conditions or cell types like metastatic and non-metastatic tumor cells.

Tissue microarrays (TMAs) allow analysis of tissue specimens at nucleic acid or protein level (Kononen et al., 1998; Avninder et al., 2008). These have vastly facilitated the clinical validation of molecular discoveries made with the aid of genomics and proteomics methods. TMAs are constructed from archival formalin-fixed paraffin embedded tissue and can contain patient follow-up data to help classify the clinical significance of the findings. In this thesis work, proteomics was used to find novel proteins that might play important roles in cancer metastasis. Furthermore, the role of one of the identified proteins, nucleophosmin (NPM), was analyzed in breast cancer by using TMAs as well as cell biological and biochemical methods.

2 REVIEW OF THE LITERATURE

2.1 Heterogeneity of cancer

Cancer refers to a collection of heterogeneous malignancies in various locations in the body. Cancer is caused by uncontrolled cell growth and resistance to cell death. Slow accumulation of alterations in proto-oncogenes, tumor-suppressor genes, DNA-repair genes and microRNA genes together with epigenetic changes in one cell or a small group of cells is considered to lead to cancer development with varying times depending on the tumor type. Recent, debatable theories suggest that not all cancer cells in a tumor are alike and that only so called cancer stem cells (CSCs) or tumor-initiating cells (TICs) would be able to maintain the tumor by possessing the ability to self-renew and proliferate. Other tumor cells would differentiate into cells that constitute the bulk of the tumor mass (Reya, 2001; Zhou, 2009). The neoplastic cancer cells harboring genetic alterations do not manifest the disease alone but form organ-like structures together with the tumor microenvironment (TME), which is composed of different types of normal stromal cells and the extracellular matrix (ECM). Consequently, cancer formation depends on both cancer cell-intrinsic pathways and cancer cell-extrinsic pathways (Hanahan, 2012).

Cancers are categorized into different types depending on their tissue of origin. Carcinomas like lung, breast and colon cancer originate from epithelial tissues and represent the most common cancer type. Non-epithelial cancers can be divided into i) sarcomas which originate from mesenchymal cells, ii) hematological cancers (leukemias and lymphomas), which originate from hematopoietic cells and iii) neuronal cancers (gliomas, glioblastomas, neuroblastomas, schwannomas, medulloblastomas) which originate from various components of the central and peripheral nervous system. Recent gene array technologies have, however, revealed a heterogeneity in tumors appearing in the same organ i.e. lung (West et al., 2012), skin (Vidwans et al., 2011) or breast (Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003; Hu et al., 2006) . This information can be used to separate the breast, skin or

lung tumors into several distinct molecular subtypes and in the future help to develop individual treatment guidelines for the different subtypes.

2.1.1 Intrinsic breast cancer molecular subtypes

Breast cancer is a heterologous group of diseases in terms of histology, therapeutic response, metastatic dissemination, and patient outcomes and it has recently been divided into the following intrinsic biological subtypes (Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003; Hu et al., 2006): Luminal A, Luminal B, basal-like breast cancer (BLBC), human epidermal growth factor receptor-2-enriched (HER2-enriched) and normal-like. Of these, Luminal A and B are positive for estrogen receptor (ER) while BLBC and HER2-enriched tumors are ER negative (Goldhirsch et al., 2011). Luminal A and B subtypes differ from each other in their HER2 expression and/or proliferation index so that luminal A tumors are HER2-negative and luminal B tumors HER2-positive. Recently, a new instrinsic, claudin low subtype of breast cancer, was also suggested (Prat et al., 2010).

Gene expression profiling is not used in clinical practice to classify tumors. Therefore, the gene array-based intrinsic subtypes have been evaluated in immunohistochemistry by using antibodies against common markers determining the subtypes: ER, progesterone receptor (PR) and HER2. In addition, epidermal growth factor receptor (EGFR) (Carey et al., 2006), cytokeratin-5/6 (CK5/6) (Carey et al., 2006; Nielsen et al., 2004; Blows et al., 2010), and markers like human epidermal growth factor receptor-1 (HER1) (Nielsen et al., 2004) or Ki67 (Hugh et al., 2009; Cheang et al., 2009) have been used to classify the basal subtype, depending on the study.

The molecular subtypes differ in their mutation status for the tumor suppressor protein p53. Only about 12-15% of luminal A tumors harbor p53 mutations while function of p53 is lost by mutation or other means in most of the BLBCs (Carey et al., 2006; Cancer Genome Atlas Network, 2012; Dumay et al., 2013). In addition to molecular subtypes, breast cancers can be classified as triple negative (TNBC), which shows negative staining for HER2,

ER and PR (Reis-Filho and Tutt, 2008). TNBCs comprise of various kinds of tumors, but majority of them are BLBCs (Carey et al., 2010). The tumor suppressor RB is commonly affected in TNBC and BLBC (Gauthier et al., 2007; Herschkowitz et al., 2008; Subhawong et al., 2009). In addition, most *BRCA1* mutant breast cancers are both triple negative and basal-like (Turner and Reis-Filho, 2006; Atchley et al., 2008; Hartman et al., 2012).

The prognosis of breast cancer patients is generally favorable and mortality has declined due to early detection and improved adjuvant therapies (Schopper and de Wolf, 2009). However, the metastatic dissemination of breast cancer to other organs is not uncommon and women with advanced disease still have a median survival time of only approximately two years (Largillier et al., 2008; Anderson et al., 2000). Currently, most of the breast cancer patients are treated with adjuvant therapy because of the lack of proper prognostic and predictive markers of metastasis. Novel markers of metastasis are needed to help clinicians to select the estimated 40% of patients that will benefit from the adjuvant therapy. In addition, the quality of life would increase for the patients that can be cured by local treatment only since they would not have to needlessly suffer from the side effects of the adjuvant therapy (Weigelt et al., 2005).

The molecular differences in breast cancer subtypes result in distinct clinical outcomes and responses to treatment; in general, the luminal A tumors associate with favorable and the BLBC and HER2-enriched tumors with poor prognosis (Carey et al., 2006; Voduc et al., 2010; Dawood et al., 2011; Arvold et al., 2011; Sorlie et al., 2001). The subtypes also have distinct preference for their metastatic sites. Luminal A cancers metastasize first to bone, HER2-enriched cancers to liver and lung and basal cancers to liver and brain (Sihto et al., 2011; Smid et al., 2008).

The biological mechanisms for breast cancer heterogeneity are mainly unknown. Possible explanations include distinct cell of origin, like CSCs or progenitor cells and tumor subtype–specific genetic events. These two mechanisms are not necessarily mutually exclusive. Two major epithelial cell populations are found in the mammary gland; the inner luminal epithelial cells and the outer (basal) myoepithelial cells, embedded in a stromal matrix.

These two populations can be divided in further sub-populations. For example, the luminal layer is a mixed population of ER positive and ER negative cells. Functionally, the mammary epithelial cells can be classified as stem, progenitor and differentiated cells. Luminal compartment contains majority of the progenitor cells while the stem cell activity is mainly found in the basal layer (Molyneux and Smalley, 2011). Mouse models (Ginestier et al., 2012) and the presence of these different cell populations in the mammary gland supports the hypothesis that breast cancer heterogeneity would result from different molecular changes occurring in different cell types (Dontu et al., 2003). Recent research indicates that BRCA1-associated breast cancers and potentially also non-familial BLBC and TNBC would originate from the luminal ER negative progenitors (Molyneux and Smalley, 2011).

2.2 Metastatic dissemination of cancer

One of the hallmarks in cancer progression is the acquisition of an invasive phenotype that allows cancer cells to spread to distant sites in the body and form metastatic lesions that are resistant to many cancer treatments (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). The term “metastasis” was invented in 1829 by a French gynecologist Recamier (Recamier, 1829) to describe the spread of cancer from its original growth sites to other parts in the body. Even today, the metastatic dissemination, rather than the primary tumor, is responsible for 90% of cancer deaths making metastasis the most serious challenge for cancer treatment (Gupta and Massague, 2006).The formation of metastasis depends on multiple steps the details of which are still poorly understood. In addition to the tumor cells TME or stroma participates in tumor progression, metastasis and response to treatment (Sleeman et al., 2012; Joyce and Pollard, 2009). In breast cancer, ECM and numerous stromal cell types, including endothelial and immune cells, fibroblasts, and adipocytes make up the primary tumor TME (Place et al., 2011).

Proteins and multiprotein complexes at the cell surface play important roles in sensing the environment, the signaling and the adhesive contacts between tumor and stromal cells during different stages of the metastatic dissemination (Karhemo et al., 2012; Place et al., 2011; Bendas and Borsig, 2012). In addition, tumor cells use their cell surface proteins to interact with platelets, leukocytes, and soluble components during the establishment of metastatic lesions (Bendas and Borsig, 2012). In the following paragraphs some examples of known cell surface proteins affecting the different parts of the metastasis process are given.

2.2.1 The metastatic cascade

Cancer cells must complete a set of well-defined, interrelated steps, globally referred to as the metastatic cascade, to develop clinically detectable metastases. Cancer cells must detach from the primary tumor, invade and survive in the lymphatics and/or blood vessels to be transported to distant organs where they must adhere, extravasate, and proliferate to form a metastatic lesion (Valastyan and Weinberg, 2011; Fidler, 2003) (Figure 1).

Due to the complexity of the metastasis cascade, metastasis is an inefficient process and can fail at any step (Chambers et al., 2002; Mehlen and Puisieux, 2006; Fidler, 1970). The entry of tumor cells into the circulation is common and more than a million cells per gram of tumor can be shed daily (Butler and Gullino, 1975). However, only a fraction of the shed tumor cells actually survive in the circulation as circulating tumor cells (CTCs) or in bone marrow as disseminated tumor cells (DTCs) (Ross and Slodkowska, 2009; Mehlen and Puisieux, 2006).

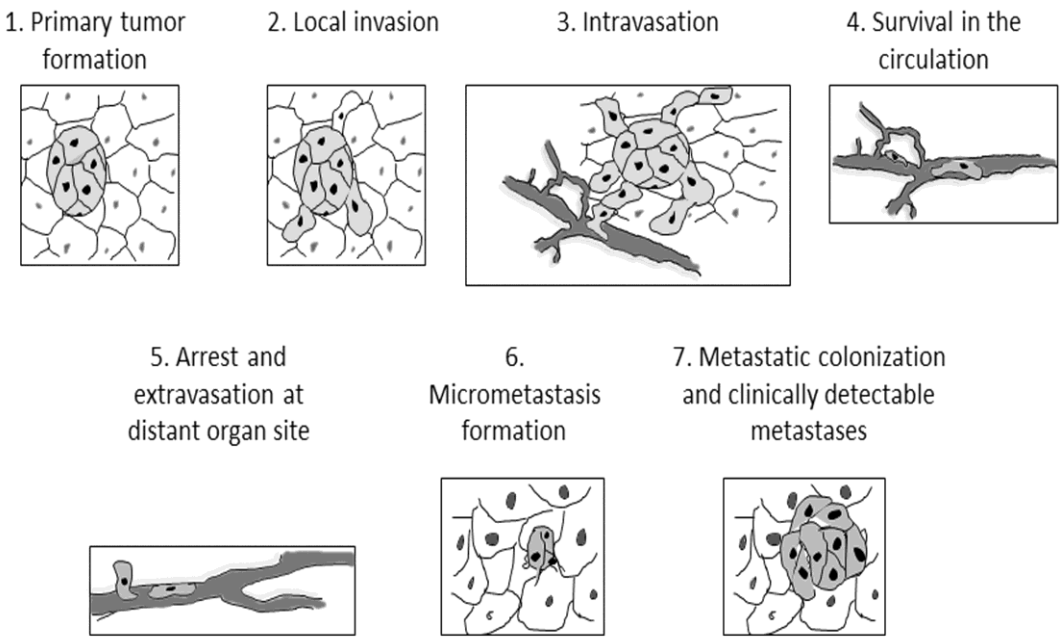

Figure 1. The Invasion-Metastasis Cascade. To be able to grow as a distant metastasis, cancer cell need to go through a series of different steps termed the invasion-metastasis cascade. The cell needs to invade the surrounding tissue (local invasion), enter the circulation (intravasation), survive in the circulation to be transported to distant sites. A cancer cell needs to be able to attach to the capillary wall at the distant organ, extravasate to form micrometastasis. Finally, to grow as a full-blown metastasis, cancer cell needs to avoid metastatic dormancy and proliferate at the distant site. (Adapted and modified from (Valastyan and Weinberg, 2011)).

Recently, it was suggested that this complex metastatic cascade could be simplified into two major phases: i) physical translocation from the primary tumor to the distant organ (contains the invasion, survival in the circulation and transport to the distant site) and ii) colonization at the distant site (Chaffer and Weinberg, 2011). The molecular mechanisms underlying the first phase are quite well resolved. During embryogenesis cells lose their epithelial characteristics and gain mesenchymal properties in a process entitled epithelial to mesenchymal transition (EMT). EMT is considered to play a role at least in the first phase of metastasis by changing the adhesive properties of tumor cells and promoting their motility, thereby increasing their invasiveness (Berx et al., 2007). However, the functional contribution of EMT to metastasis in patients is still debated (Sleeman et al., 2012).

At the cell surface perspective, deregulated expression of many cell surface ECM remodeling enzymes like heparanases and matrix metalloproteinases is a common event in human cancers to help cancer cells in their invasion (Lu et al., 2011). In most tumors cell surface proteins and protein complexes like integrins, syndecans, dystroglycans, immunoglobulin superfamily cell adhesion proteins, cadherins, and hyaluronan binding proteins (hyaladherins) like CD44 participate in cell–tissue interaction and migration during invasion (Gritsenko et al., 2012). Recently, microvesicles, small membrane-enclosed sacs, shed from tumor cells have been shown to participate in the regulation of the ECM invasion and evasion of the immune response (Muralidharan-Chari et al., 2010).

The mechanisms playing a role in the second phase of metastasis, colonization still remains largely unknown. In experimental models survival and proliferation at the secondary site, have been shown to be highly inefficient (Allan et al., 2006). Clinically, it would be of utmost importance to better understand the second phase to be able to treat patients who have already developed metastasis at the time of diagnosis (Chaffer and Weinberg, 2011). Most likely, cell surface proteins play a major role also in this phase.

The models mentioned above describe metastasis as a unidirectional process, where cancer cells from the primary tumor seed metastasis in distant sites. Interestingly, based on recent experimental models a new

metastasis concept, termed self-seeding, has been proposed. This paradigm considers metastasis as a multidirectional process whereby cancer cells can seed distant sites as well as the primary tumor itself (Norton and Massague, 2006; Comen et al., 2011).

2.2.2 Organ specific colonization and microenvironment

The distribution of full-blown metastases to different organs is not random and different tumor types disseminate and form metastatic lesions in a different set of organs (Auerbach et al., 1987; Johnson et al., 1991; Nguyen et al., 2009). Already in 1889 Stephen Paget proposed, based on his analysis of autopsy records from 735 breast cancer patients, that DTCs, or "seeds," would only colonize organ microenvironments, or "soils," that wwould be compatible with the growth of the DTCs (Paget, 1889). This so called seed and soil hypothesis suggested that outcome of metastasis depends on the interactions between the tumor cells and host tissue, a fact that is currently emerging as a critical determinant of metastasis (Lorusso and Ruegg, 2012).

The seed and soil hypothesis was challenged by Ewing by stating that organ specificity is accounted by mechanical forces and circulatory patterns between the primary tumor and the secondary site (Ewing, 1928). Later, Fidler and coworkers (Fidler and Kripke, 1977; Hart and Fidler, 1980) revealed that although CTCs in the vasculature traffic through all organs, metastases selectively develop in organs with suitable environment. In support of this, breast cancer frequently metastasizes to the lungs, bones and liver (Largillier et al., 2008) and brain (Palmieri et al., 2006), which do not have a direct circulatory connection to breast tissue (Lorusso and Ruegg, 2012).

Mechanistically, networking of cytokines and chemokines expressed in the target tissue and interacting with their cognate receptors expressed on the surface of the tumor cells is involved in organ specificity highlighting the important role of cell surface proteins in this process. As examples, production of osteoclast-activating factors such as pTHRp, Il-11, Il-6, tumor necrosis factor-α (TNFα), and granulocyte–macrophage colony stimulating

factor (GM-CSF) is required for the ability of breast cancer cells to form bone metastases (Nguyen et al., 2009).

Recent evidence from cell line and animal studies suggests that specific cell surface adhesion molecules on tumor cells and their receptors on the lung endothelium mediate breast cancer cell adhesion and extravasation in the lung (Lu and Kang, 2007). In breast cancer, the CXCR4 and CCR7 receptors, expressed on breast cancer cell surface and their ligands CXCL12 and CCL21 on the organs, might play a role in cancer cell arrest and migration into secondary organs (Muller et al., 2001; Wang et al., 2006). Metadherin, which is overexpressed on the surface of metastatic breast cancer cells, mediates targeting of tumor cells specifically to the lung, but not to other organs through binding to an unknown receptor expressed in lung endothelium (Brown and Ruoslahti, 2004). In addition, interaction of cell surface fibronectin with a dipeptidyl peptidase IV (DPP IV, (Cheng et al., 1998) and cell surface expression of α6β4 integrin and its adhesion to human CLCA2 protein (Abdel-Ghany et al., 2001) were shown to participate in the lung metastasis of breast cancer.

As described, the successful engraftment and growth of cancer cells in distant organs depends on a receptive microenvironment. Recent evidence points out that growth factors and other molecules secreted by the primary tumor could prime certain tissues for tumor cell engraftment by forming a pre-metastatic niche (Psaila and Lyden, 2009; Psaila et al., 2006). The location of these pre-metastatic niches would subsequently determine the organs in which metastases will form (Sleeman and Cremers, 2007).

2.2.3 Dormancy of tumor cells

Metastases can occur after long latency periods that range from years to decades after the primary treatment (Chaffer and Weinberg, 2011). For example, about 45% of breast cancer patients will relapse and develop distant metastases years or decades after the diagnosis (Karrison et al., 1999). This prolonged time observed for the development of distant, metastatic disease

indicates a period of dormancy before DTCs are able to grow into clinically relevant metastases (Castano et al., 2011).

In an experimental model, dormant tumor cells have been observed in metastasis free organs of animals carrying spontaneously metastatic primary tumors (Suzuki et al., 2006) suggesting that dormancy might also affect organ specific colonization so that in the hostile environments DTCs undergo dormancy while in a receptive environment the same cells are able to grow into macrometastases.

Concept of tumor cell dormancy was introduced already in 1934 by an Australian pathologist Rupert Willis (Willis, 1934). Despite the early observation of cancer dormancy, not much is known about cancer cells during this period of dormancy and what awakens them even thought this is clinically a very important question (Uhr and Pantel, 2011). Dormant cells may be reactivated by modification of their microenvironment (Barkan et al., 2010; Barkan et al., 2010) or by loss of metastasis suppressor genes, which are defined by their ability to inhibit overt metastasis in a secondary organ without affecting tumor growth at the primary site (Horak et al., 2008).

Two forms of dormancy, which are not mutually exclusive, have been suggested (Castano et al., 2011). Dormancy might be accounted for by a mitotic arrest of tumor cells (Naumov et al., 2002; Goodison et al., 2003; Muller et al., 2005), which is called cellular dormancy (Castano et al., 2011). In another form of dormancy, called tumor dormancy, the rate of cell death counterbalances the rate of cell proliferation within a tumor mass (Meng et al., 2004; Naumov et al., 2006). However, the mechanisms controlling the size of the tumor cell population are unknown (Meng et al., 2004) but it might be kept constant by some of the same mechanisms that control the size of normal organs (Uhr and Pantel, 2011).

The molecular mechanisms of dormancy have mostly been studied in experimental models. Dormant state might be regulated by a crosstalk between tumor cell surface proteins and the ECM at the secondary sites (Barkan et al., 2010). As examples, cell surface urokinase receptor (Aguirre Ghiso et al., 1999; Liu et al., 2002; Heiss et al., 1995; Aguirre-Ghiso et al., 2001) and integrin β1-focal adhesion kinase (FAK) signaling axis has been

shown to regulate metastatic dormancy in three dimensional *in vitro* model (Shibue and Weinberg, 2009; Pontier and Muller, 2008). Maintenance of the dormant state might also be affected by failure of angiogenesis (Goss and Chambers, 2010). Recently, Kim et al. revealed a dormancy signature in breast cancer and showed that ER-positive tumors, which generally associate with favorable prognosis and carry a dormancy signature, are likely to undergo prolonged dormancy before resuming metastatic growth (Kim et al., 2012).

2.2.4 When tumor cells acquire their metastatic capability?

To understand the properties of metastatic tumor cells and target the process, it is important to resolve, when the tumor cells acquire their full metastatic capability and are able to go through both phases of metastasis. Two basic models have been suggested to represent the timing of the metastatic cascade during tumor progression. The prevailing, so called linear progression model, suggests that a rare subpopulation of tumor cells in the primary tumor acquires a full malignant phenotype via genomic alterations at late stages of primary tumor development. These fully malignant cells would have all the properties required for the formation of metastatic lesions (Fidler and Kripke, 1977; Klein, 2009; Poste and Fidler, 1980). The association of large tumor size with higher frequency of metastases (Koscielny et al., 1984), the correlation between primary tumor size and risk of lymph node and distant metastasis (Comen et al., 2011), the curative effect of surgery on smaller lesions (Klein, 2009) and the variable metastatic capability of different murine B16 melanoma cell clones (Fidler and Kripke, 1977) support the linear progression model.

A recent, parallel model of metastasis questions the linear progression model and proposes that metastases arise from DTCs, which do not necessarily disseminate near the end of primary tumor development and acquire their fully metastatic phenotype independent of the primary tumor (Klein, 2009). Quantitation of human cancer growth rates demonstrates that the metastatic lesions must have been initiated long before the diagnosis of

the primary tumor (Collins et al., 1956; Friberg and Mattson, 1997; Klein, 2009). Furthermore, the long interval between diagnosis and relapse with metastatic, distant disease in breast cancer, e.g. dormancy, supports the parallel progression model (Nguyen et al., 2009). In addition, genetic comparison of breast cancer metastases to their matched primary tumors supports the parallel progression and early onset of metastases (Sleeman et al., 2012; Kuukasjarvi et al., 1997; Torres et al., 2007; Santos et al., 2008). In addition, recent studies show that breast cancer derived DTCs display fewer genetic alterations than their corresponding primary tumors (Schardt et al., 2005; Schmidt-Kittler et al., 2003) and these alterations do not resemble those detected in the corresponding primary tumors (Lorusso and Ruegg, 2012; Schmidt-Kittler et al., 2003; Braun et al., 2005). However, DTCs are currently isolated for analysis by the aid of certain epithelial markers, which might not be expressed by all DTCs such as tumor cells undergoing EMT hampering the reliable comparison of all DTCs to primary tumors (Sleeman et al., 2012).

It has also been suggested that particular early oncogenic events that drive primary tumor growth might give cancer cells their propensity to metastasize as opposed to the theory that metastasis arises from rare cells accumulating metastasis specific genomic alterations in time (Bernards and Weinberg, 2002; Ramaswamy et al., 2003). Another explanation for the metastatic paradox was revealed from metastatic studies in different genetic background in mice. These preliminary studies indicate that the host genetic background has a significant role in determining the metastatic potential early in oncogenesis (Hunter et al., 2003).

2.3 Subclones of MDA-MB-435 cancer cell line as a metastasis model

2.3.1 Origin of the parental MDA-MB-435 cell line?

The MDA-MB-435 cell line was created from malignant cells in a pleural effusion of a 31-year old Caucasian woman with breast cancer. Patient had an extensive infiltrating breast carcinoma and two of the eight axillary lymph nodes contained breast cancer cells. She died one year after her diagnosis because of a metastatic disease (Cailleau et al., 1978; Brinkley et al., 1980).

The origin of the cell line has later been questioned. The microarray data and karyotyping show that the cell line has a gene expression pattern most compatible with melanocyte origin and identical to the M14 melanoma cells that were used as a feeder cell line during the establishment of the MDA-MB-435 cell line (Ross et al., 2000; Rae et al., 2004; Rae et al., 2007). In support of the melanoma origin, the MDA-MB-435 cells were shown to express RXRG, TYR, ACP5, and DCP genes, which are commonly transcribed in melanocytes but not in various commonly used breast cancer cell lines (Ellison et al., 2002).

However, MDA-MB-435 cells can be induced to express breast differentiation markers and secrete milk lipids (Sellappan et al., 2004). They also express a number of breast and epithelial cell specific proteins together with melanocytic features, most likely due to lineage infidelity (Sellappan et al., 2004; Nerlich and Bachmeier, 2013). It is possible that the MDA-MB-435 cells represent undifferentiated breast cancer and express melanocytic differentiation markers since primary breast tumors have been shown to express melanocyte related genes (Montel et al., 2009). Furthermore, based on karyotype and allelotype, the MDA-MB-435 cells are of female origin and cannot therefore be classified as M14 melanoma, which originate from a male patient (Chambers, 2009; Hollestelle and Schutte, 2009).

In respect to the breast cancer molecular subtypes, MDA-MB-435 cell line has been classified to represent the basal subtype (Neve et al., 2006; Chavez

et al., 2010). It contains a wild-type BRAC1 (Elstrodt et al., 2006) and a mutant p53 (Hollestelle et al., 2010; O'Connor et al., 1997).

2.3.2 Cloning and characterization of the non-metastatic and metastatic subclones of the MDA-MB-435

Limiting dilution technique, with direct microscopic monitoring of monocellular origin, has been used to create a pair of isogenic clones of the MDA-MB-435 cell line. Screening for the metastatic ability in athymic mice revealed that these clones significantly differ in in their metastatic capability (Urquidi et al., 2002). Both the metastatic and non-metastatic cells are able to reach the lungs of tumor-bearing mice thus capable of going through the first stage of metastasis, the physical translocation, while only the metastatic cells can perform the second phase of metastasis, colonization, in lungs and form full-blown metastatic lesions (Goodison et al., 2003). In addition, metastatic cells that formed lung metastases could be observed in a dormant state in other organs (Suzuki et al., 2006). Thus, these cell lines enable the comparative investigation of cellular and molecular events necessary for the second phase of metastasis and for the maintenance and subsequent release from dormancy at the secondary sites in a stable and isogenic model.

2.3.3 Identification of metastasis related cell surface proteins

As described earlier, the details and molecular mechanisms of metastasis are not fully resolved. At the late stages of metastasis blood flow and other mechanical factors influence the delivery of cancer cells to specific organs, whereas molecular interactions between the cancer cells and the organ influence the probability that the cells will proliferate and grow as a metastatic lesion at the new site. Cell surface proteins, the proteins protruding from the plasma membrane into the extracellular space, are important mediators of these interactions (Place et al., 2011; Bendas and Borsig, 2012; Karhemo et al., 2012). Cell surface molecules also represent two-thirds of the current protein-based drug targets (Hopkins and Groom,

2002; Overington et al., 2006). Some, but not all, cell surface proteins can be classified as plasma membrane proteins. For example, ligands bound to their surface receptors can be regarded only as cell surface proteins because they lack direct contact with the plasma membrane.

Heterogeneity of tumors and presence of stromal cells within tumors hamper the search for cancer cell specific metastasis-associated proteins (Hondermarck et al., 2008). Large-scale analysis of cell surface proteins is hindered by the poor solubility of hydrophobic, integral membrane proteins. Cell surface and membrane proteins are also of low abundant and difficult to detect without enrichment or fractionation. Most cells can be removed from tissues, but this is difficult to perform without perturbing the cell surface (Leth-Larsen et al., 2010). For these reasons, it is difficult to study these proteins *in vivo* at tissue level.

The use of isogenic cell lines differing in their metastatic and dormant behavior enables identification and functional analysis of candidate proteins affecting tumor cell dormancy and metastasis. Cultured cancer cells are easy to expand and fractionate and currently provide the best source for the analysis of metastasis-associated cell surface proteins in cancer cells. The drawback of these models is the lack of proper microenvironment, which has been shown to play a crucial role in metastasis. Therefore, expression results obtained from the cell line models need further validation in animal models and in clinical samples. In addition, mechanistic analyses are required for in depth understanding on how these molecules affect the metastatic process.

Various methods including density gradient centrifugation and numerous chemical labeling techniques have been described for the isolation and enrichment of the cell surface and/or plasma membrane proteins for proteomic analyses (Elschenbroich et al., 2010; Leth-Larsen et al., 2010; Cordwell and Thingholm, 2010). Due to their accessibility, cell surface proteins of intact cells can be tagged with a membrane-impermeable biotin on amino acid residues located in extracellular space, which allows exploitation of the extraordinarily stable and non-covalent interaction between avidin and biotin in isolation and detection of the biotinylated cell surface proteins. Several commercial chemical biotinylation reagents, which

vary in their biotin moiety, spacer and reactive moiety, have been developed (Elia, 2008).

Importantly, by using labeling methods, all proteins accessible for the labeling reagent e.g., ligands bound to their receptors are isolated and analyzed with downstream applications. When adherent cell cultures are used as starting material, ECM proteins and secreted proteins bound to their ligands or ECM can also be labeled and isolated. Finally, the isolated cell surface proteins can be quantified and identified by proteomics methods to revela differentially expressed proteins.

2.4 Nucleophosmin; oncogene, tumor suppressor or both?

Nucleophosmin (NPM, B23, numatrin, NO38, hereafter referred as NPM) is a ubiquitously expressed multifunctional nucleolar phosphoprotein involved in a complex network of biological activities related to both growth suppression and proliferation. Importantly, it seems that deregulation in NPM homeostasis is often observed in tumors. NPM expression and gene integrity are frequently altered in human cancers and it has been attributed both tumor suppressive and oncogenic functions (Grisendi et al., 2006).

The human *NPM1* gene maps to chromosome 5q35 and contains 12 exons. Alternative splicing of the *NPM1* transcript results into two isoforms, B23.1 and B23.2 (Wang et al., 1993). In the full length B23.1 exon 9 is spliced to exon 11. The coding sequence stops at exon 12 resulting in a protein containing 294 amino acid residues. The shorter B23.2 isoform consists of 259 amino acids and lacks 35 amino acids at the C-terminus as a result of splicing the exon 9 to exon 10, which contains a stop codon. B23.2 is present in cells at low levels (Wang et al., 1993). Circular dichroism spectral analysis revealed similar secondary structures (mainly of beta-sheet and beta-turns) in B23.1 and B23.2 (Umekawa et al., 1993). Recently a third splice variant that lacks an internal exon 8 has been identified in the human EST database (GenBank accession number: NM_199185).

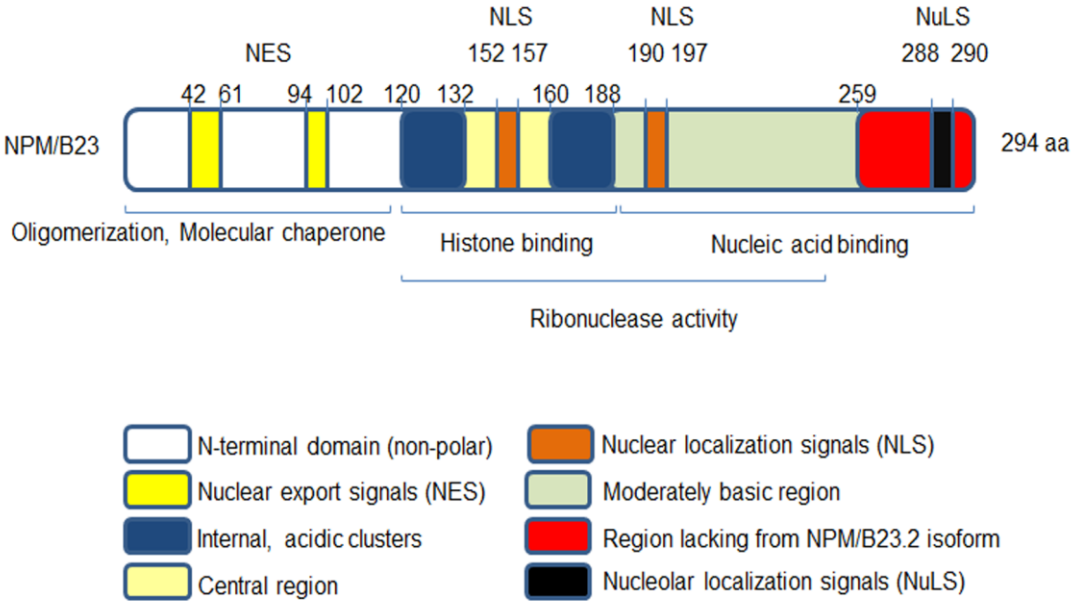

Figure 2. Functional motifs and domains of NPM/B23. NPM is composed of a non-polar N-terminal domain containing nuclear export signal (NES). This part of the protein is important for oligomerization and molecular chaperone function. The central part of the protein contains a two acidic clusters required for histone binding . The C-terminal domain is needed for nucleic acid binding and together with the central region posesses ribonuclease activity. Adapted and modified from:(Grisendi et al., 2006).

The functional motifs and domains of NPM are shown in Figure 2. NPM contains motifs for nucleolar localization (Nishimura et al., 2002), nuclear import (Hingorani et al., 2000) as well as for nuclear export (Yu et al., 2006; Wang et al., 2005). It has distinct N- and C-terminal domains and a central part containing two acidic clusters important for NPM's histone binding (Okuwaki et al., 2001) and ribonuclease activity (Hingorani et al., 2000). Importantly, the C-terminal domain of NPM interacts with multiple proteins, like p53 (residues 249–262 of NPM) (Lambert and Buckle, 2006), c-Myc (residues 187–259 of NPM) (Li et al., 2008), FOXM1 (residues 187-259 of NPM) (Bhat et al., 2011) and Akt (residues 239-294 of NPM) (Lee et al., 2008). In addition, the C-terminal domain (residues 260-294 together with Thr199 and Thr234/237) is shown to be responsible for the binding of NPM to the phosphorylated RB (Takemura et al., 1999; Lin et al., 2010). Residues in parenthesis refer to the different fragments of NPM used to determine the binding site. Therefore, the actual binding site might be shorter. In addition,

NPM has been reported to bind to a number of other proteins involved in cellular processes like DNA replication, transcription, and repair, cell cycle control, ribosome biogenesis, viral replication, apoptosis, stability and splicing of mRNA, protein modification, mitotic spindle, cytoskeleton, and centromeres.

Notably, most of the functional data on NPM is supported by biochemical and *in vitro* data only. A future challenge is to understand the mechanisms which control NPM activation and recruitment to distinct subcellular sites and protein complexes to carry out its pleiotropic, often seemingly antagonistic, biological functions. The emerging picture indicates that NPM's biological roles are tightly regulated by several mechanisms such as NPM's expression level, localization, oligomerization status, post-translational modifications and NPM binding partners. Cellular distribution of NPM is closely associated with phospho/dephosphorylation events (Yun et al., 2003) and the nucleolar localization of NPM has been reported to require adenosine-5'-triphosphate (ATP) (Chang et al., 1998; Choi et al., 2008; Choi et al., 2008).

NPM undergoes several post-translational modifications, which regulate its cellular localization and function. A recent *in silico* analysis predicted that contains 40 potential phosphosites which would be substrates of at least 38 kinases. Based on the associated kinases the authors suggested that NPM phosphorylation is related to cellular processes such as apoptosis, cell survival, cell proliferation, and response to DNA damage stimulus (Ramos-Echazabal et al., 2012), all functions previously attributed to NPM. Post-translational modifications of NPM are summarized in table 1.

The following paragraphs summarize the current knowledge on NPM regulation and functions relevant to this thesis work.

Table 1. Post-translational modifications of NPM

Enzyme	Modification	Timing	Relevance	Ref.
Plk2	phosphorylation (serine 4)	S-phase	Centriole duplication	(Krause and Hoffmann, 2010)
GRK5	phosphorylation (serine 4)		sensitivity of cells to PLK1 inhibition	(So et al., 2012)
Unknown kinase	phosphorylation (serines 10 and 70)		Regulation of CDK1 activity	(Du et al., 2010)
CK2	phosphorylation (serine 125)	interphase	Dissociation from nucleolus	(Negi and Olson, 2006)
ATR	phosphorylation (serine 125)		Inhibition of the UV-induced p53 phosphorylation at Ser15	(Maiguel et al., 2004)
Cyclin B-CDK1	phosphorylation (threonines 199, 219, 234, 237)	mitosis	Dissociation from the nucleolus, RNA binding, centrosomal association	(Peter et al., 1990; Okuwaki et al., 2002; Negi and Olson, 2006; Cha et al., 2004)
Cyclin E-CDK2	phosphorylation (threonine 199)	G1	Centrosome cycle control	(Okuda et al., 2000; Tokuyama et al., 2001)
Cyclin A-CDK2	phosphorylation (threonine 199)	S-phase and G1	Centrosome cycle control	(Tokuyama et al., 2001)
Cyclin D-CDK4	phosphorylation (threonine 199)	G1	Centrosome cycle control	(Adon et al., 2010)
Viral cyclin V-CDK6	phosphorylation (threonine 199)		Kaposi,s sarcoma herpesvirus latency	(Sarek et al., 2010)

Enzyme	Modification	Timing	Relevance	Ref.
PKC	phosphorylation (serine 225)		Phosphorylation *in vitro* in nuclear membrane fraction	(Beckmann et al., 1992)
Nek2A	phosphorylation	mitosis	Centrosomal localization	(Yao et al., 2004)
PP1β	Dephosphorylation (threonines 199, 234, 237)		pRB binding and consequent E2F1-dependent DNA repair	(Negi and Olson, 2006; Lin et al., 2010; Haneji et al., 2012)
SUMO-1 and SUMO-2	Sumoylation		NPM localization, ribosome biogenesis	(Tago et al., 2005; Liu et al., 2007; Haindl et al., 2008)
SENP3 and 5	Desumoylation		Control of ribosome biogenesis	(Haindl et al., 2008; Yun et al., 2008)
p300	Acetylation mostly on the C-terminal domain)		Stimulation of chromatin transcription	(Swaminathan et al., 2005)
BRCA1-BARD1 ubiquitin ligase	ubiquitinylation	mitosis	Stabilization of NPM	(Sato et al., 2004)
Not reported	poly-(ADP-ribosyl)ation			(Ramsamooj et al., 1995)

Plk2 = Polo-like kinase, GRK5 = G protein-coupled receptor kinase 5, CK2 = Casein kinase 2, ATR = Ataxia telangiectasia mutated and Rad3 Related protein kinsase, PKC = Protein kinase C, PP1 β = Protein phosphatase 1 β, SUMO = Small ubiquitin-like modifier, SENP = SUMO1/sentrin/SMT3 specific peptidase

2.4.1 Discovery of NPM as nucleolar protein involved in ribosome assembly and transport

NPM was originally designated as B23 based on its position on a two dimensional gel analysis from acid-extracted nucleolar proteins isolated from normal rat liver and Novikoff hepatoma ascites cells (Orrick et al., 1973). It was shown to be a highly expressed nucleolar phosphoprotein (Kang et al., 1974; Prestayko et al., 1974), which localizes especially to the granular region of the nucleolus, a site where ribosomes are assembled (Spector et al., 1984). In addition, NPM was proved to be identical with a nuclear protein numatrin, which is tightly bound to nuclear matrix (Feuerstein and Mond, 1987; Feuerstein et al., 1988). NPM's nucleolar localization and its shuttling between the nucleus and cytoplasm (Borer et al., 1989) led to the earliest proposal that it facilitates ribosome assembly (Dumbar et al., 1989) and transport of pre-ribosomal particles (Yung et al., 1985). Later NPM has been shown to direct the nuclear export of both 40S and 60S ribosomal subunits (Maggi et al., 2008). According to current knowledge, NPM provides the necessary export signals and chaperoning capabilities that are required to transport ribosome components from nucleus to cytoplasm and by these functions it seems to balance protein synthesis to cell growth and proliferation (Grisendi et al., 2006; Falini et al., 2007), important determinants of cancer growth.

2.4.2 NPM as a chaperone and its role in transcriptional regulation

Genomic DNA is compacted into chromatin by organizing it into nucleosomes by its association with four histone proteins H2A, H2B, H3, and H4. The correct assembly/disassembly of nucleosomes is mediated by histone chaperones whose precise function is necessary for DNA-dependent activities like transcription, replication and repair (Burgess and Zhang, 2013).

NPM is biochemically defined as a member of the nucleoplasmin/nucleophosmin family of nuclear chaperones. The family

members share an acidic core-domain at the N-terminus of the protein which is responsible for oligomer formation and chaperone activity (Schmidt-Zachmann et al., 1987; Hingorani et al., 2000; Frehlick et al., 2007; Okuwaki et al., 2001; Prinos et al., 2011). Crystal structure of the N-terminal core domain in NPM revealed that it forms pentamers which can further oligomerize into decamers similar to the other nucleoplasmin family members (Lee et al., 2007). In addition to the N-terminal domain, some C-terminal regions might be required for NPM oligomer formation (Liu and Chan, 1991). A recent computational analysis of the N-terminal domain in NPM proposes that NPM monomer-oligomer status can be regulated by transformation from a folded, pentameric structure to a monomeric, disordered state through phosphorylation events (Mitrea and Kriwacki, 2012).

As a nuclear chaperone NPM can bind to histones and assemble nucleosomes in vitro (Okuwaki et al., 2001; Gadad et al., 2011). NPM possesses chaperone activity also for proteins since it can prevent aggregation and thermal denaturation of proteins such as HIV-1 Rev protein, liver alcohol dehydrogenase and carboxypeptidase A *in vitro* (Szebeni and Olson, 1999).

NPM's histone chaperone activity is enhanced by p300 mediated acetylation on the C-terminal domain which modulates *in vitro* transcription from chromatin templates by RNA Pol II (Swaminathan et al., 2005). Furthermore, acetylated NPM localizes to nucleoplasm where it regulates transcriptional activation of genes implicated in oral cancer manifestation such as tumor necrosis factor alpha and interleukin-6 receptor (Shandilya et al., 2009). NPM negatively regulates a histone-modifying enzyme GCN5 and thus transcription and this regulation was enhanced by phosphorylation of NPM at Thr199 (Zou et al., 2008). Recent results indicate that the NPM might regulate gene expression at specific G-quadruplex regions (Xu et al., 2007; Federici et al., 2010; Gallo et al., 2012), which are common in oncogene promoters, whereas a reduced frequency is observed in tumor suppressor genes (Qin and Hurley, 2008). NPM has been shown to regulate transcription, either positively or negatively, also through interaction with

several transcriptional regulatory partners like oncogenic transcription factor Forkhead box M1 (FOXM) (Bhat et al., 2011), androgen receptor (Leotoing et al., 2008), activating protein transcription factor 2 (Liu et al., 2007) and YY1 (Inouye and Seto, 1994), to mention some. Intriguingly, YY1 has also been indicated to regulate NPM gene expression (Chan et al., 1997).

NPM associates with both DNA and RNA with its C-terminal nucleic acid binding domain (Wang et al., 1994; Hingorani et al., 2000; Okuwaki et al., 2002) and it has been reported to have endoribonuclease activity to ribosomal RNA (rRNA) (Savkur and Olson, 1998; Hingorani et al., 2000). NPM binds to rRNA chromatin and regulates the histone density around rRNA genes (Murano et al., 2008). This activity requires NPM's RNA binding activity and is regulated by its cell cycle-dependent phosphorylation (Hisaoka et al., 2010).

The B23.2 splice variant of NPM lacks the C-terminal domain and therefore is not able to bind double stranded DNA (Wang et al., 1994) and has lower ribonuclease activity than the full-length NPM protein (Herrera et al., 1995). In addition, B23.2 can heterodimerize with the full length NPM and reduces its RNA-binding (Okuwaki et al., 2002). Oligomer formation by itself has also been shown to decrease NPM's DNA binding (Herrera et al., 1996).

2.4.3 Molecular pathways regulated by NPM: RB, ARF-p53-Mdm2 pathway and c-Myc

The retinoblastoma protein (RB), encoded by the *RB1* gene, is the first known human tumor suppressor (Knudson, 1984; Corson, 2007; Dimaras, 2008; Huang et al., 1988) which is inactivated in multitude of solid cancers by various distinct mechanisms (Burkhart and Sage, 2008). In normal quiescent tissues RB maintains cell cycle arrest by repressing the activity of E2F-family of transcription factors (E2F1, E2F2, and E2F3) (Burkhart and Sage, 2008; Cobrinik, 2005). This repression is relieved by mitogenic or oncogenic signals, which induce RB phosphorylation (pRB) (Mittnacht, 1998). As a

consequence, the expression of genes involved in DNA-replication, mitosis and cytokinesis is induced (Markey et al., 2007).

Hyperphosphorylated RB translocates into nucleoli in late S or G2 phase. Translocation is mediated by NPM (Takemura et al., 1999; Takemura et al., 2002), most likely after dephosphorylation of NPM on threonines 199, 234, 237 by PP1β (Lin et al., 2010). The biological significance of the pRB-NPM complex and nucleolar localization is unclear but RB and NPM have been shown to synergistically stimulate DNA polymerase alpha activity (Takemura et al., 1999).

The p53 tumor supressor gene is defective in about half of all tumors, regardless of their type or origin (Hollstein et al., 1991; Levine et al., 1991) and in the remaining cancers a considerable number has alterations in the p53 pathway. The p53 gene encodes a tetrameric protein that functions mainly as a transcription factor at the crossroads of cellular stress response pathways (like DNA damage, oncogene activation and hypoxia) controlling the expression of genes involved in cell division and viability, growth arrest and apoptosis (Levine and Oren, 2009). NPM regulates p53 by directly binding to it (Colombo et al., 2002; Maiguel et al., 2004) and by affecting the p53 regulatory proteins ARF (Bertwistle et al., 2004; Korgaonkar et al., 2005) and Mdm2 (Kurki et al., 2004). NPM has a dual effect on p53 and it can either stabilize (Colombo et al., 2002; Horn and Vousden, 2004; Kurki et al., 2004) or inhibit p53 (Li et al., 2004; Wu et al., 2002; Dhar and St Clair, 2009; Li et al., 2007) and apoptosis. Mechanisms regulating such opposite effects are mainly unknown. NPM oligomerization might be necessary for its capability to inhibition p53-mediated apoptosis (Qi et al., 2008; Jian et al., 2009). NPM could also inhibit p53 activity by competing with p53 phosphorylation (Maiguel et al., 2004; Nalabothula et al., 2010). NPM binding partners have also been reported to regulate its effect on p53 (Ji et al., 2012; Fukawa et al., 2012).

The small tumor suppressor protein p14ARF (humans, p19ARF in mouse, hereafter referred as ARF) functions as a tumor suppressor by inhibiting the Mdm2 mediated degradation of p53. It binds directly to Mdm2 in a site distinct from the p53 binding domain (Ashcroft and Vousden, 1999) which

inhibits the ubiquitin ligase activity of Mdm2 (Honda and Yasuda, 1999). Even though the complex interaction between ARF and NPM has been under extensive investigation over the last years, it is still not fully understood. NPM binds ARF in the nucleoli in a quantitative manner (Bertwistle et al., 2004). ARF mutants lacking the NPM binding site are unstable (Kuo et al., 2004) and ARF localizes to nucleoplasm in MEFs lacking both p53 and NPM (Colombo et al., 2005) indicating that NPM stabilizes ARF in the nucleoli. On the other hand, ARF has been shown to mediate NPM degradation (Itahana et al., 2003) indicating a feed-back loop between these two proteins. ARF has also been shown to block NPM function in rRNA processing and the transport of pre-ribosomal particles (Savkur and Olson, 1998; Itahana et al., 2003; Sugimoto et al., 2003; Brady et al., 2004). NPM is also a target for ARF induced sumoylation (Tago et al., 2005).

c-Myc induces both p53-dependent and p53-independent apoptosis upon up-regulation of the tumor suppressor ARF. However, when overexpressed or deregulated c-Myc becomes oncogenic (Hoffman and Liebermann, 2008; Nilsson and Cleveland, 2003). Importantly, in animal models, most if not all, c-Myc-induced tumors have inactivated the ARF-p53 pathway (Eischen et al., 1999; Nilsson and Cleveland, 2003).There is a complex interaction between NPM and the c-Myc-oncogene. Expression of c-Myc correlates with NPM expression (Guo et al., 2000; Neiman et al., 2001; Kim et al., 2000) and NPM is a transcriptional target of c-Myc (Zeller et al., 2001). In addition, NPM directly interacts with c-Myc and regulates c-Myc mediated rDNA transcription, nucleolar localization (Li and Hann, 2013) and expression of c-Myc target genes like eIF4E (Li et al., 2008). NPM overexpression has been shown to enhance c-Myc-induced proliferation and transformation in $p53^{-/-}$ $ARF^{-/-}$ double knockout (DKO) mouse embryo fibroblasts (MEFs) (Li et al., 2008)

Recent studies, however, have shown that NPM binds to the G-quadruplex DNA on c-Myc gene-promoter which suppresses c-Myc gene expression (Siddiqui-Jain et al., 2002; Gallo et al., 2012). In addition, Reduction in NPM levels accelerated lymphomagenesis in μ-Myc transgenic mice (Grisendi et al., 2005).

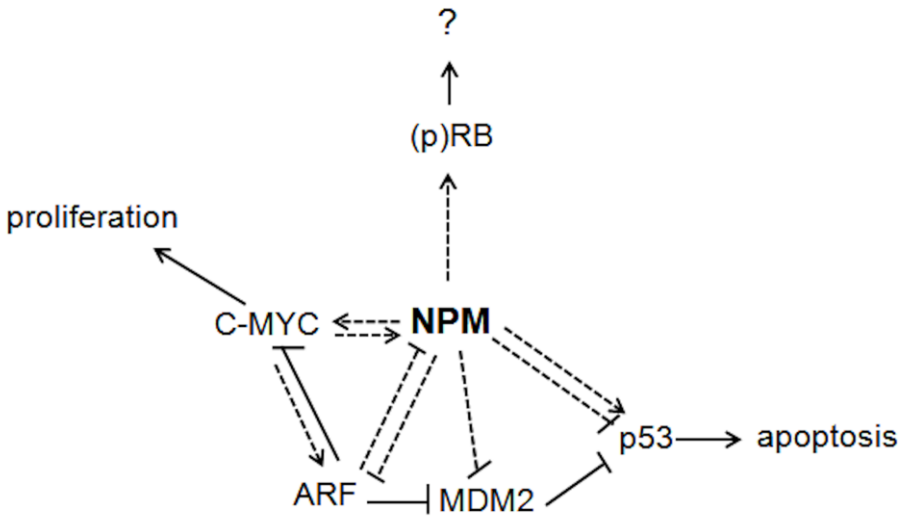

Figure 3. Schematic representation of the discussed pathways controlled by NPM. Solid lines indicate connections with strong scientific backround.

2.4.4 Role of NPM in centrosome duplication

Centrosomes, the microtubule-organizing centers of animal cells, are mainly composed of mother and daughter cylindrical microtubule-based centrioles and a matrix of associated pericentriolar material. Centrosome is duplicated in coordination with DNA replication. Centrosome amplification is frequently observed in cancers and is postulated to be one cause of chromosome instability. Proper centrosome function is also required for cytokinesis (Wang et al., 2004; Zyss and Gergely, 2009).

Cultured NPM-null cells or mice carrying a single inactivated *Npm1* allele display increased centrosome numbers (Grisendi et al., 2005). In accordance, NPM is shown to control centrosome cycle. NPM attaches to single, unduplicated centrosomes in late G1 (Ochs et al., 1983) and re-associates with the centrosomes during mitosis (Zatsepina et al., 1999; Okuda et al., 2000) at least partly after its phosphorylation on Thr234 and Thr237 by cyclin B-CDK1 complex (Cha et al., 2004). At the centrosome, NPM localizes between the centriole pair (Shinmura et al., 2005). Cyclin E(A)-CDK2 (Tokuyama et al., 2001; Okuda et al., 2000) and cyclin D-CDK4 phosphorylate NPM at Thr199, which partly dissociates NPM from the

centrosome subsequently allowing separation of the centrioles and centrosome duplication (Okuda et al., 2000; Tokuyama et al., 2001; Okuda, 2002; Tarapore et al., 2002).

Some NPM phosphorylated on Thr199 remain attached to centrioles, translocates toward a mother centriole (Shinmura et al., 2005) and binds to and activates centrosomal ROCK II kinase, which further promotes centrosome duplication (Ma et al., 2006). In addition to Thr199 phosphorylation, Plk2-mediated phosphorylation of NPM on serine 4 has been shown to promote centrosome duplication (Krause and Hoffmann, 2010). However, while important in regulating the correct centrosome cycle, the Thr199 phosphorylation might not be necessary for NPM's growth promoting functions (Brady et al., 2009).

2.4.5 NPM in human tumors and in mice

NPM protein levels are elevated in rapidly dividing cells (Feuerstein and Mond, 1987; Feuerstein and Mond, 1987; Nozawa et al., 1996) and its expression increases rapidly in the early G1 phase during mitosis (Feuerstein et al., 1988) which led to the proposed growth promoting function of NPM. In accordance, NPM levels decrease in cells that are withdrawn from the cell cycle or undergoing apoptosis (Hsu and Yung, 1998; You et al., 1999; Yung, 2004). NPM expression associates with poor prognosis, recurrence or lymph node metastasis in Ewing's sarcoma (Kikuta et al., 2009), bladder carcinoma (Tsui et al., 2008), colon cancer (Liu et al., 2012) and oral squamous cell carcinoma (Coutinho-Camillo et al., 2010) in a relative small patient materials (n=31-154). In hematological malignancies NPM is involved in chromosome translocations, where its N-terminal oligomerization domain is fused with different partners like ALK, RARα and MLF. NPM oligomerization domain contributes to tumor development by activating the oncogenic potential of the fused protein partner (Grisendi et al., 2006).

On the contrary, Bocker and coworkers did not find difference in NPM expression between normal and malignant epithelial prostatic cells (Bocker

et al., 1995) and NPM expression did not correlate with patient prognosis in an analysis of 55 melanoma patient samples (Calli et al., 2011). No amplification of *NPM1* has been detected in human tumors, but deletion of the gene has been observed in non-small cell lung carcinomas (Mendes-da-Silva et al., 2000). In addition, *NPM1* is the most frequently mutated gene in acute myeloid leukemia (AML) accounting for ~35% of cases (Falini et al., 2005). Mutations in the C-terminal domain lead to mutant proteins that aberrantly and stably localize to the cytoplasm due to an additional nuclear export signal at the C-terminal domain (Bolli et al., 2007; Albiero et al., 2007). Mutant NPM oligomerizes with the wild type protein, which is also transported to the cytoplasm thus functioning as a dominant negative (Falini et al., 2009). These mutations seem to represent tumor –initiating lesions, since they appear before the other AML-associated genetic alterations can be detected (den Besten et al., 2005).

Npm1 acts as a haploinsufficient tumor suppressor in the hematopoietic compartment in mice (Sportoletti et al., 2008). Inactivation of *Npm1* in the germ line leads to embryonic lethality in mice at midgestation indicating indispensable role during development (Colombo et al., 2005; Grisendi et al., 2005). *Npm1* null embryos display genomic instability, widespread apoptosis, and activation of p53. In addition, cultured NPM-null cells or mice carrying a single inactivated *Npm1* allele accumulate DNA damage (Colombo et al., 2005) and have increased centrosome numbers (Grisendi et al., 2005). In addition, $Npm1^{+/-}$ MEFs have an immortal phenotype with noticeably high proliferation rates (Colombo et al., 2005; Grisendi et al., 2005) and display accelerated lymphomagenesis when crossed with μ-Myc transgenic mice (Grisendi et al., 2005).

3 AIMS OF THE STUDY

Cell surface proteins are known to be important players in regulating cancer metastasis. As the molecular mechanisms underlying the second phase of metastasis (colonization at the distant site) and metastatic dormancy are poorly resolved, we wanted to identify novel cell surface proteins contributing to colonization at the distant site. The aim was not to gain a comprehensive picture of the cell surface proteins of the two cell lines used but to identify differentially expressed proteins for future studies.

The specific aims included:

- Isolate the cell surface proteins from invasive, non-metastatic and fully metastatic cell clones of the MDA-MB-435 cell line for a proteomic based comparative study
- Profile the differential expression of cell surface proteins in order to discover interesting, differentially expressed proteins for future functional studies in metastatic colonization
- Analyze the breast cancer associated role and function of one of the identified proteins, nucleophosmin, in more detail

4 MATERIALS AND METHODS

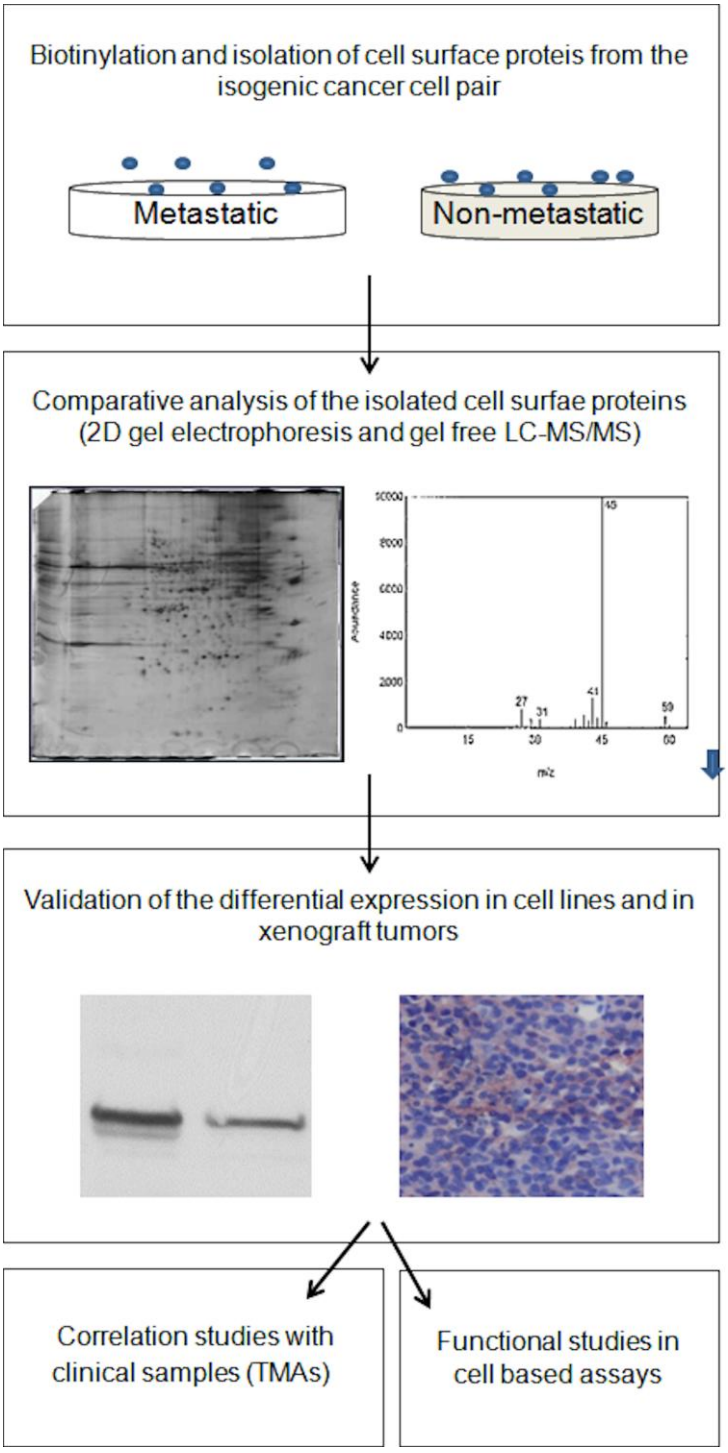

Figure 4. Flowchart of the study

The materials and methods of this study are listed in table 2. with detailed descriptions in the following text and in the original publications, here referred to by Roman numerals.

Table 2. Methods used in the study

Methods	Used in
Cell proliferation assays	II, III
Cell surface biotinylation	I, unpublished data
Cloning	II, III
Creation of stable cell lines	II, III
Gene knockdown by RNA interference	III
Immunofluorescence microscopy	I, II, III, unpublished data
Immunohistochemistry	I, II, III
Immunoprecipitation	II, III
Implantation of tumor cells into mice	I
Lentiviral production	II
Mammalian cell culture	I, II, III
Mass spectrometry based protein identification	I, unpublished data
Network and pathway analyses	II
Quantitative real-time PCR	II
RNA extraction	II, III

Methods	Used in
Scoring of tumor microarrays	II, III
SDS-PAGE and immunoblotting	I, II, II, unpublished data
Sequence analysis	II, III
Soft agar assay	II
Statistics	I, II, III
Transduction of mammalian cells	II
Transfection of mammalian cells	II, III
3D cell culture	II
17β-Estradiol Treatment	II

4.1 Cell culture (2D and 3D), cell treatments, cell proliferation and soft agar assays

Human cancer cell lines (Table 1) were cultured at + 37 °C in 5% CO_2 atmosphere in appropriate medium. For 3D growth/invasion assays cross-linked fibrin gels were prepared by combining 75 µl plasminogen-free human fibrinogen (6 mg/ml; Calbiochem) in Hank's Balanced Salt Solution (HBSS) and 75 µl HBSS (pH 7.4) containing 4 U/ml human thrombin and 400 µg/ml aprotinin (both from Sigma-Aldrich). 5000 cells were suspended in 40 µl of the prepared, cross-linked fibrin and transferred to a 24-well plate. The plate was incubated for 1 h at 37°C to allow complete gelling. The cells were grown for 4–16 days, fixed and photographed

For the 17β-Estradiol Treatment cells were starved for 48 hours in Phenol Red-free RPMI (Gibco, California, USA) supplemented with 2% charcoal-dextran filtrated fetal calf serum (HyClone; Thermo Scientific, Logan, UT), 1% glutamine, and penicillin-streptomycin. Starved cells were treated with 10 nmol/L 17β-estradiol for 3 days and analyzed.

Cell proliferation was measured by growing cells on 96-well plates for indicated time periods. Cells were incubated 2 hours with 10 µL MTT (5 mg/mL) and lysed (10% SDS, 10 mmol/L HCl) overnight. Absorbance was measured at 540 nm using Multiskan Ascent software version 2.6 (Thermo Labsystems, Vantaa, Finland).

For Soft agar assays tissue culture dishes containing a 2-mL layer of solidified 0.7% agar in a complete medium were prepared. Cells (5×10^3 cells per 35-mm well) were suspended in complete medium containing 0.35% agarose and plated on top of the solidified bottom agar. After 14 days, number of colonies was quantified. For visualization, foci were methanol-fixed and stained with 0.005% crystal violet.

4.2 Cell surface protein isolation

Cells were grown 3 days after which they were washed three times with Dulbecco (PBS + 0.901 mM $CaCl_2$ + 0.492 mM $MgCl_2$) and labeled with EZ-Link Sulfo-NHS-SS-Biotin (Pierce, Rockford, IL, USA; 0.5 mg/ml in) for 30-minutes in Dulbecco on ice. Labeling solution was removed by washing the cells twice with Dulbecco followed by blockade of the non-reacted biotin with 20 mM glycine for 15 minutes and washed with Dulbecco + 100 µM oxidized glutathione (Sigma-Aldrich, St. Louis, MO). Cells were lysed and the membrane proteins solubilized in 500 µl of lysis buffer (PBS + 2% NP-40, 1% Triton X-100, 10% glycerol, 100 µM oxidized glutathione, EDTA free protease inhibitor tablet (Roche, Mannheim, Germany)) for 30 minutes. The cell extracts were incubated with 30 U of DNase (22 °C 50 min, Roche, Mannheim, Germany) and centrifuged for 20 min (20 800 ×*g*, at + 4 °C). Equal amounts of protein from each cell extract, determined by DC- (Bio-Rad, Hercules, CA, USA) or BCA- (Thermo Fisher Scientific, Pierce Rockford, IL, USA) protein assays were used for cell surface protein isolation. The supernatant was pre-cleared using biotin agarose beads (ImmunoPure® Immobilized D-biotin, Pierce, Rockford, IL, USA) and incubated with magnetic streptavidin beads (Dynabeads®MyOne™ Streptavidin, Invitrogen Dynal, AS, Norway). Beads were washed four times with the lysis buffer, four times with 300 mM NaCl in lysis buffer and twice with 50 mM Tris–HCl, pH 7.8. and the isolated proteins were eluted with 50 mM DTT in 50 mM Tris–HCl, pH 7.8 at 30 °C, followed by pooling of the eluates.

4.3 Mass spectrometry, protein identification, network and pathway analyses

For LC–MS/MS analysis proteins were digested with trypsin (sequence grade modified, Promega, Madison, WI, USA) and peptides were loaded to a reversed phase pre-column (NanoEase Atlantis dC18, 180 µm × 23.5 mm, Waters, Milford, MA, USA) with 0.1% formic acid followed by separation in reversed phase analytical column (PepMap 100, 75 µm × 150 mm, Thermo Fisher Scientific Dionex, Germerin, Germany) with a linear gradient (4–50%) of 95% acetonitrile in 0.08% formic acid in 35 min. Full scan for eluting peptides was acquired in an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific Inc.) introduced to via the ESI Chip interface (Advion BioSciences Inc.) in the positive-ion mode. The mass range of 300–2000 m/z on the Orbitrap-detector with 60 000 resolution (FWHM) at 400 m/z, the AGC target was set to 200 000 and the maximum inject time to 800 ms. Six MS/MS data-dependent scans were acquired on LTQ based on full scan, with the AGC target set to 10 000 and the maximum inject time set to 100 ms. An isolation width of 2 m/z was used for precursor selection. Peptide fragmentation was done in normalized collision energy of 35%, activation time of 100 ms and activation Q set to 0.25. Precursors, whose charge state couldn't be determined or charge state was + 1, were discarded from the MS/MS analysis. Precursors were dynamically excluded for 10 s with a repeat count of 1. Both full scan and MS/MS scans consisted of one microscan and they were acquired as profile data.

Mascot (version 2.2, in-house server), Swissprot 2010 (516 080 sequences, 181 677 051 residues) and the following parameters were used for protein identification: enzyme: trypsin, peptide mass tolerance: 0.02 Da, fragment mass tolerance 0.8 Da, max missed cleavages 2, variable modifications: carbamidomethy (C), carboxymethyl (C), deamidated (NQ), oxidation (M), propionamide (C), and pyridylethyl (C). Same search was also conducted limiting the taxonomy to human, except using variable modifications carbamidomethyl (C), oxidation (M), and propionamide (C). Both results were combined and the results filtered to human proteins. Mascot score > 40 was required for identifications in the total protein list. In addition, at least one unique peptide was required for the reported differentially expressed proteins. Pathway and network analyses for the differentially expressed proteins were performed in a computational platform Moksiskaan.

4.4 Immunoprecipitation and immunoblotting

Equal amounts of protein from post nuclear supernatans of each extract were pre-cleared using protein G sepharose™ 4 fast flow beads (GE Healthcare Bio-Sciences Ab, Uppsala, Sweden) followed by incubation with the appropriate antibodies (+ 4 °C, o/n). The antibody-antigen complex was captured on G sepharose beads (+ 4 °C, 30 min), which were washed four times with lysis buffer and boiled in *Laemmli* buffer.

Proteins were separated using SDS-PAGE and transferred to Immobilon-P Membrane (Millipore, Bedford, MA, USA). Immunoblots were incubated with the appropriate primary and secondary antibodies (HRP conjugated, Dako, Glostrup, Denmark) followed by enhanced cemiluminecense visualization using the SuperSignal West Pico kit (Thermo Fisher Scientific, Pierce, Rockford, IL, USA).

4.5 Immunohistochemistry and immunofluorescence analyses

For immunofluorescence staining of intracellular antigens, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% NP-40 in PBS. Unspecific antibody binding sites were blocked using 3% bovine serum albumin in PBS. The cells were incubated with primary and secondary (Molecular Probes goat anti-mouse Alexa Fluor 594; Invitrogen, Carlsbad, CA) antibodies. For staining of cell surface antigens live cells were blocked and incubated with the primary antibodies on ice after which they were fixed and incubated with the secondary antibodies. DNA was visualized with DAPI (Vector Laboratories, Burlingame, CA).

For the immunohistochemical analyses paraffin embedded sections were deparaffinized and the antigens retrieved with the appropriate method for each antibody. Primary antibodies were detected by using the TSA-kit (Perkin Elmer, Waltham, MA, USA) and the signal was visualized with the AEC-reagent (Sigma-Aldrich, St. Louis, MO) or by using peroxidase staining (biotinylated horse anti-mouse immunoglobulins 1:200 and Vectastain ABC complex; Vector Laboratories), which was visualized with 3,3′-diaminobenzidine (Vector Laboratories). Images were captured with an Olympus DP50 camera and with Olympus Studio Lite software version 1.0 or 1.01. For the C-kit staining we used PowerVision Novocastra preantibody blocking solution (Leica Microsystems, Wetzlar, Germany) for primary antibody incubation. Staining was detected using a PowerVision + Poly-HRP histostaining kit (DPVB + 110DAB; ImmunoVision Technologies, Daly City,

CA; Springdale, AR) according to the manufacturer's instructions. A Ventana Discovery IHC slide stainer (Ventana Medical Systems, Tucson, AZ) and a Ventana 3,3′-diaminobenzidine tetrahydrochloride biotin avidin detection kit was used for the cytokeratin staining.

4.6 Patients and classification of breast cancer subtypes in the TMAs

We used two different breast cancer patient cohorts, the FinProg and FinHer, which both contained detailed clinical information allowing survival and association analyses. The cancers were classified into five biological subtypes as follows; luminal A (ER+ and/or PR+, HER2-), luminal B (ER+ and/or PR+, HER2+), basal-like (ER-, PR-, HER2-, CK5+ and/or EGFR+), HER2-enriched (HER2+, ER-, PR-), and non-expressor type (negative for all five key classifiers). The non-expressor type corresponds to unclassifiable triple-negative breast cancer. An ethics committee at Helsinki University hospital approved the FinHer study. Regarding FinProg material permission to use formalin fixed paraffin embedded tissues for research purposes was provided by the Ministry of Social Affairs and Health, Finland.

4.7 Statistical analyses

In the TMAs the associations between factors were tested with the $\chi 2$ test. The odds ratio was used to examine the strength of the relationships. Kaplan-Meier method was applied to calculate the life tables. Multivariate survival analyses were performed with the Cox proportional hazards model, entering the appropriate covariates.

Progenesis LC–MS software was used to quantitate the expression differences in the comparative proteomic analysis. The runs were divided into following 4 groups; metastatic and non-metastatic non-biotinylated controls and metastatic and non-metastatic samples. The peptides present in control groups, determined by manual comparison of the peptides, were discarded form the analysis followed by comparison of the metastatic and non-metastatic samples against each other. ANOVA and q-values calculated by the Progenesis software were used to deduce differentiating peptides. The Progenesis stats-package was used to perform unsupervised principal component analysis (PCA).

5 RESULTS AND DISCUSSION

5.1 Comparative analysis of metastasis and dormancy associated cell surface proteins (I and unpublished data)

As described earlier, the details and molecular mechanisms of metastasis are not fully resolved. Therefore, we wanted to identify novel cell surface proteins contributing to the formation of metastasis, especially to the second phase (colonization at the distant site). In this thesis work we used metastatic and non-metastatic subclones of the MDA-MB-435 human carcinoma cell line as a model for the metastatic spread of cancer. The non-metastatic cells leave the primary tumor as efficiently as the metastatic ones and are detected as single cells in the lung capillaries. However, they fail to go through the latest steps of the metastatic cascade and cannot form full- blown metastases (Goodison et al., 2003).

Originally, this thesis work aimed at comparing the differences in cell surface proteins between the metastatic and non-metastatic cell pair by using two-dimensional (2D) gel electrophoresis. (Karhemo et al., unpublished data). However, the fast development of gel-free shotgun proteomics technologies (Duncan et al., 2010) and mass spectrometry based protein quantification (Ong and Mann, 2005; Domon and Aebersold, 2010) led us to analyze and quantify our isolated cell surface samples using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Study I).

5.1.1 Optimized method for isolation of biotinylated cell surface proteins (I)

For comparative proteomic analysis the cell surface proteins of the isogenic non-metastatic and metastatic cell pair were labeled with biotinylation reagents that react with primary amines (-NH2), such as the side-chain of lysine followed by isolation with magnetic streptavidin beads.

Studies used as reference to optimize the isolation of biotinylated cell surface proteins reported a consistent identification of intracellular proteins (Scheurer et al., 2005; Roesli et al., 2008). To diminish this intracellular background the cell extracts were treated with DNAseI to dissolve the cytosolic actin and proteins associated with it from the viscous DNA in order to liberate a maximal amount of membrane proteins. In addition, samples were pre-cleared by using biotin agarose beads and intracellular background was controlled by including non-biotinylated control samples in the analysis.

The gel-free LC-MS/MS approach resulted in identification of 86 proteins in total from both cell lines. This relatively limited number of identifications might be explained by performance of database search within the Progenesis LC–MS software after exclusion of all peptides present in the control samples, by the low amount of starting material and the performance of the LC–MS analyses without exclusion lists to exclude already fragmented features.

Importantly, more than 60% of the identified proteins localized to the cell surface/extracellular space according to the Finnish Red Cross in house Cell Surface Protein Classifier. The specific enrichment of cell surface proteins was further verified by analyzing the presence of a cell surface protein, cluster of differentiation 109 (CD109), the endoplasmic protein BiP, the Golgi protein Golgin 97, the subunit IV of mitochondrial protein cytochrome C oxidase and an intracellular protein Erk1/2 in the cell surface and unbound fractions (representing proteins that did not bind to the streptavidin beads) in a Western blot analysis. Our optimized protocol specifically isolated the cell surface proteins since that fraction was free of organelle contaminants (Figure 1A and B in publication I).

5.1.2 Cell surface protein changes in the metastatic cells (I, unpublished data)

This thesis work revealed 29 differentially expressed proteins (Table 3) from the cell surface samples between the metastatic and non-metastatic cells using the 2D gels (Karhemo et al, unpublished results) and the LC-MS/MS analyses (publication I). Six differentially expressed proteins were identified from silver stained 2D gels based on visual comparison of the spot intensities (Table 3). The gel-free analysis revealed 23 differentially expressed proteins (Table 3, Table 1 in publication I).

Based on a Uniprot database (http://www.uniprot.org/) annotation and literature search 19/29 proteins (66%) represented proteins with known cell surface localization. The gel-free LC-MS/MS based approach proved to be superior in identifying cell surface membrane proteins compared to the 2D gels. Only one protein, LGALS3, identified using the 2D gels is reported to be secreted to the cell surface and only one protein is predicted to contain a single transmembrane domain. On the contrary, 18/23 (78%) of the differentially expressed proteins identified in the gel-free analysis represented proteins with previously reported cell surface localization and 15 were either single or multi-pass transmembrane proteins (Table 3).

Interestingly, most of the identified proteins (20/29, 69%) were overexpressed in the metastatic cells. Similar results have been obtained in a previous proteomic analysis of ultracentrifuged and density separated plasma membrane fractions of the same isogenic MDA-MB-435 cancer cell pair (Lund et al., 2009). These results indicate that overexpression/localization of certain proteins on the surface of the metastatic cells may facilitate extravasation and colonization of the metastatic cells by adapting them to the new microenvironment at the secondary site.

Table 3. Differentially expressed proteins identified in the thesis work

Name	Relative expression (metastatic/non-metastatic) in LC-MS/MS	Method	Cellular localization[A]	Type of membrane protein
MRPL12		2D	mitochondrion, ribosome	
RPLP0		2D	cytoplasm, ribosome	
MRPL39		2D	mitochondrion, ribosome	
NPM		2D	nucleolus, nucleus	
SSR4		2D	endoplasmic reticulum membrane	single-pass
LGALS3		2D	secreted, cytoplasm, nucleus	peripheral
HLA-DRB1	8.8	LC-MS/MS	cell membrane	single-pass
HLA-DRA1	2.53	LC-MS/MS	cell membrane	single-pass
BST2	7.88	LC-MS/MS	cell membrane, trans-Golgi network	single-pass, GPI-anchor
PTGFRN	7.44	LC-MS/MS	cell membrane	single-pass
ART3	6.03	LC-MS/MS	cell membrane	GPI-anchor
PROCR	4.4	LC-MS/MS	cell membrane	single-pass
GPR56	3.01	LC-MS/MS	cell membrane	multi-pass
ADAM10	2.74	LC-MS/MS	cell membrane	single-pass
PTPRF	2.4	LC-MS/MS	cell membrane	single-pass
ATP1B3	2.21	LC-MS/MS	cell membrane	single-pass
CD109	2.11	LC-MS/MS	cell membrane	GPI-anchor

Name	Relative expression (metastatic/non-metastatic) in LC-MS/MS	Method	Cellular localization[A]	Type of membrane protein
ITGA6	2.08	LC-MS/MS	cell membrane	single-pass
TUBA1C	2.01	LC-MS/MS	cytoplasm, cytoskeleton	
IGSF8	2	LC-MS/MS	cell membrane	single-pass
ITGAV	1.82	LC-MS/MS	cell membrane	single-pass
HSPD1	1.76	LC-MS/MS	mitochondrion matrix	
FASN	1.7	LC-MS/MS	cytoplasm	
ITGB1	1.44	LC-MS/MS	cell membrane	single-pass
GOT2	0.43	LC-MS/MS	mitochondrion matrix, cell membrane	peripheral
NME1	0.49	LC-MS/MS	cytoplasm, nucleus	
CHL1	0.56	LC-MS/MS	cell membrane	single-pass
CD97	0.73	LC-MS/MS	cell membrane	multi-pass
ALDH7A1	0.75	LC-MS/MS	mitochondrio, nucleus	

Overexpressed, **underexpressed** (metastatic/non-metastatic)
[A] UniProt and literature
Proteins that displayed ≥ 2-fold in their expression are underlined, proteins whose differential surface expression was validated by antibodies are highlighted.

Previous gene array analysis, which compared a subclone of the original metastatic MDA-MB-435 clone to the non-metastatic one, revealed overexpression of vesicular trafficking related genes (Rab27, Rab38, VAMPA and syntaxin 7) in the metastatic cells. Rab27 and Rab38 are small GTPases functioning at various stages of vesicular fusion and trafficking. The vesicle-associated membrane protein-associated protein A (VAMPA) and syntaxin 7 mediate the fusion of organelle membranes and vesicle attachment to

lysosomes, (Montel et al., 2005; Steeg, 2005). Overexpression of these proteins could partly explain the observed upregulation of cell surface proteins in the metastatic cells. However, when using the cell surface biotinylation method, detergents used to solubilize the membrane proteins influence the type of proteins that will be identified with the downstream applications as only the proteins soluble in the detergent will be isolated and identified. Other comparatice proteomic analyses of biotinylated cell surface fractions from paired non-metastatic and metastatic cells have reported different ratios (Conn et al., 2008; Roesli et al., 2009; Luque-Garcia et al., 2010). In this thesis work, only one detergent combination (2% NP-40, 1% Triton X-100, 10% glycerol) was used and therefore many proteins insoluble in this buffer were naturally excluded from the analysis. The number of differentially expressed proteins could therefore be increased by using various different detergents, which might also affect the relative numbers of up and downregulated proteins between the cell pair.

5.1.3 Validation of the differential cell surface expression of selected proteins (I, unpublished data)

In order to validate the differential expression and/or localization of the identified proteins we used Western blot and immunofluorescence analyses. In regards of the LC-MS/MS results, higher expression of PROCR (endothelial protein C receptor), CD109 and ITGA6 (integrin-α6) on the surface of the metastatic cells was confirmed (Figure 3A and B in publication I). Integrin β1 (ITGB1), which displayed only a 1.4-fold expression difference in the LC-MS/MS quantitation was used as a control. According to Western blot and immunofluorescence analyses it was expressed at equal levels at the cell surface of the cell pair (Figure 3A and B in publication I). In addition, the differential expression of two proteins, SSR4 and NPM, which were over- and under expressed at the metastatic cell surface in the 2D analysis, respectively, was validated in the cell surface samples. (Figure 5A, Karhemo et al, unpublished results).

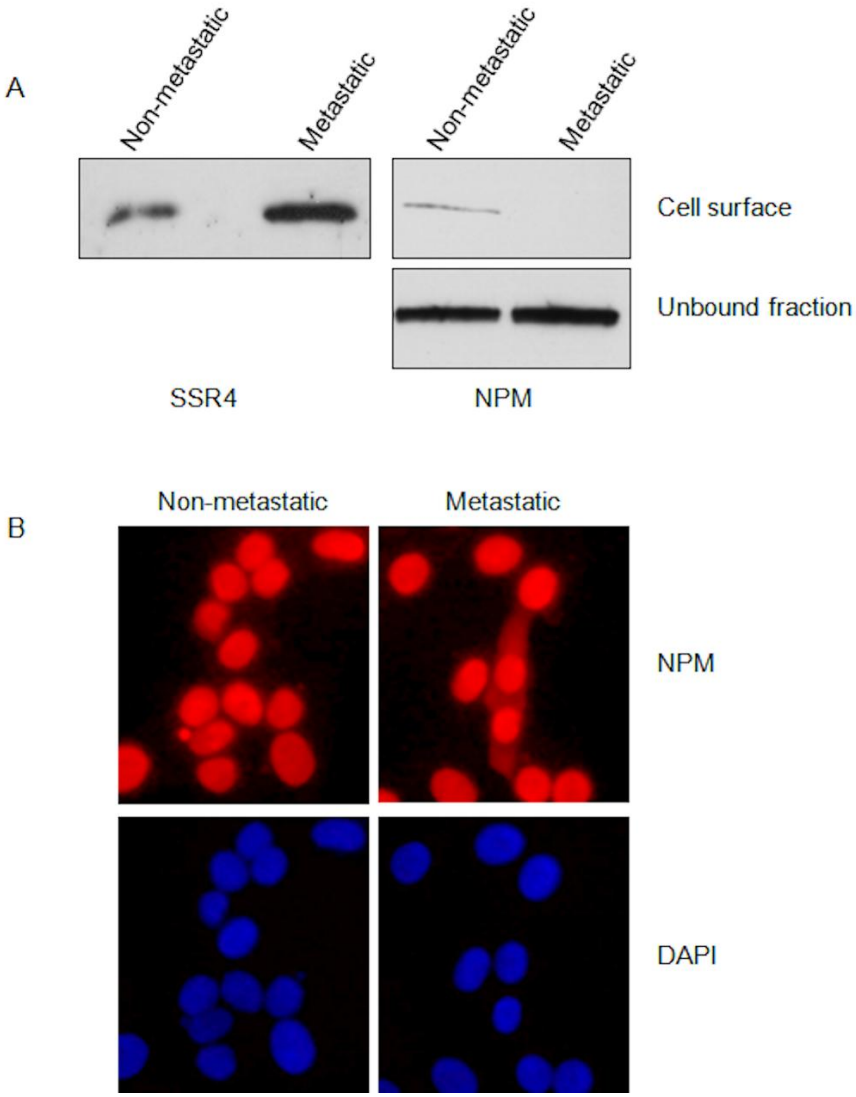

Figure 5. Analysis of SSR and NPM expression in the non-metastatic and metastatic cells. A) Expression of SSR4 and NPM in the metastatic and non-metastatic cells was detected using Western blot analysis of cell surface extracts. Expression of NPM was also analyzed from the unbound fraction representing intracellular NPM levels. B) Immunofluorescence analysis of NPM using antibodies against NPM followed by an Alexa-594 conjugated secondary antibody. Non-metastatic and metastatic cells showed equal nuclear NPM staining. However, the metastatic cells displayed an additional cytoplasmic staining in some cells. Nuclei were visualized with DAPI (blue). Images were digitally cropped in Photoshop CS4.

Since the differential expression at the cell surface could result either from differences in the expression levels or from differential localization of proteins we analyzed the expression of CD109 and PROCR in whole cell extracts and NPM in the unbound fractions. No difference in the expression level of NPM was detected (Figure 5A, Karhemo et al., unpublished results) while both PROCR and CD109 were expressed at higher levels in the

metastatic cell line compared to the non-metastatic cells (Figure 3A in publication I).Importantly, this indicates that a change of NPM localization might play a role in metastatic colonization. Metastasis associated decrease in cell surface expression of NPM has also been reported in another cell surface proteomics study (Roesli et al., 2009). To analyze NPM cellular localization in more detail in our isogenic cell lines, we stained fixed and permeabilized cells with anti-NPM/B23 antibodies. Interestingly, in both cell lines NPM/B23 displayed strong nucleoplasmic staining and in the metastatic cell line an additional cytoplasmic localization was also observed (Figure 5B, Karhemo et al., unpublished results). As seen from Figure 5A, only a minor fraction of total cellular NPM was observed at the cell surface and is most likely below the detection limit of immunofluorescence.

5.1.4 The overexpression of cd109 and ITGA6 marks cells derived from melanoma metastasis (I)

The 3-dimensional (3D) architecture provided by the ECM at both primary and secondary sites, have a profound influence on the functional properties of tumor cells (Joyce and Pollard, 2009; Sleeman et al., 2012). Importantly, the *in vitro* observed expression differences of CD109, PTGFRN, PROCR, and ITGA6 (table 3) were also detectable *in vivo* in xenograft tumor samples derived from the metastatic and non-metastatic MDA-MB-435 cells (Figure 4 in publication I).

To investigate if the differential expression of CD109 and ITGA6 could differentiate metastatic cells from non-metastatic ones in other cell models, their expression was studied in six melanoma cell lines (WM164, WM165, WM793, WM852, G361, and Bowes) and in four invasive (MDA-MB-231, BT549, HS578T, SUM159) and four non-invasive (MCF-7, ZR75-1, BT474, and T47D) breast cancer cell lines (Neve et al., 2006). CD109 is a GPI-linked cell surface protein, which negatively modulates TGFB1 signaling in keratinocytes (Finnson et al., 2006; Hagiwara et al., 2010). Expression of its transcript has previously been linked to melanoma in a transgenic melanoma mouse model (Ohshima et al., 2010) and it is expressed in BLBC (Hasegawa

et al., 2008). Shedding of CD109 by mesotrypsin has been shown to promote the malignant growth of breast cancer cells (Hockla et al., 2010) but its role in metastasis is unknown.

ITGA6 dimerizes with ITGB4 and the complex functions primarily as an adhesion receptor in normal epithelia, often as a component of adhesive structures termed hemidesmosomes. The A6B4 complex has been shown to play a role in migration, invasion and survival of carcinoma cells, most likely by alterations in the localization of A6B4 that influence its signaling capacity (Lipscomb and Mercurio, 2005).

In line with the original proteomic study, CD109 was highly expressed on the surface of the cell lines derived from metastatic melanoma (WM164, WM165, and WM852) and on the surface of WM793 cells derived from an invasive advanced vertical growth phase of melanoma. Cells derived from non-metastatic primary melanomas (G361 and Bowes) expressed negligible amounts of the protein. ITGA6 was expressed in WM852 and WM793 cells while the rest of the cell lines showed very low or undetectable levels of ITGA6 (Figure 5 in publication I, control staining in Supplemental Figure S2A in publication I). In accordance with its expression in the melanoma cell lines, three out of four invasive breast cancer cell lines (BT549, HS578T, and SUM159) expressed high levels of CD109 while none of the non-invasive breast cancer cell lines expressed detectable amounts of CD109 (Supplemental Figure S1 in publication I).

To typify the fibrin-rich tumor–stroma interface of melanomas *in vivo,* the expression of CD109 and ITGA6 was further analyzed in three-dimensional (3D) matrix composed of cross-linked fibrin (Wojtukiewicz et al., 1990). Importantly, when the cells were implanted inside a 3D matrix, both ITGA6 and CD109 were highly expressed in the cells derived from melanoma metastasis (Figure 6 in publication I, control staining in Supplemental Figure S2D in publication I). WM793 cells from advanced vertical growth phase primary melanoma also expressed detectable levels of CD109 in the 3D culture conditions while ITGA6 was essentially undetectable in all the cells derived from primary melanomas (Figure 6 in publication I).

Importantly, these results demonstrate the validity of the used cell line model and the proteomics approach. The results were validated *in vivo* and in models other than originally used. Therefore, the functional role of C109 and other identified proteins in metastatic colonization and dormancy should be studied in the future.

5.1.5 Interactome analysis indicates a role for a tetraspanin protein complex in metastasis (I)

The role of only about one-third of the significantly differentially expressed proteins identified in the gel free LC-MS/MS analysis (23 proteins) is well documented in cancer progression and metastasis. To put our novel metastasis-associated proteins into biological context we analyzed how the proteins identified in the LC-MS/MS analysis (Table 3 and Table 1 in I) interacted with each other, and which signaling pathways they were involved in using the computational platform Moksiskaan (Laakso and Hautaniemi, 2010). A two-fold expression difference was used as a threshold for up or down regulated proteins. Proteins that showed less than 2-fold expression differences were defined as stable (Table 1 in I and Table 4). To simplify the pathways and interactomes the HLA-molecules were excluded from the analyses resulting in 14 differentially expressed proteins and seven stably expressed proteins. Differential expression of HLA-molecules in this cell model has been validated in other studies (Leth-Larsen et al., 2009; Rasmussen and Ditzel, 2009).

The known relationships between the candidate proteins were analyzed in the Moksiskaan software based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Figure 2 in publication I) (Kanehisa et al., 2010). The KEGG pathways supporting these relationships included metastasis-associated pathways, such as gap junctions, focal adhesions, and insulin signaling pathway (Supplemental information S2, metastasis associated proteins p. 5 in study I).

The insulin signaling pathway is represented in the analyses via two proteins upregulated at the surface of the metastatic cells; fatty acid synthase

(FASN) and receptor-type tyrosine-protein phosphatase F (PTPRF, LAR). Excess cellular lipid level has previously been associated with cancer aggressiveness (Tsubura et al., 2009). Hedegaard et al. demonstrated higher unsaturated fatty acid levels in the metastatic MDA-MB-435 cell clone relative to the non-metastatic one (Hedegaard et al., 2010). A comparative gene expression analysis revealed significantly higher expression of fatty acid desaturase 1, which catalyzes the change of saturated fatty acids to unsaturated ones, in the metastatic MDA-MB-435 cells compared to the non-metastatic ones (Montel et al., 2006). This evidence indicates a role of fatty acids in the regulation of the metastatic potential of this cell pair.

PTPRF is a protein tyrosine phosphatase (PTP) that has been reported to regulate insulin receptor phosphorylation and signaling (Goldstein et al., 1998). The extracellular region of PTPRF contains a cell adhesion molecule-like receptor region and it localizes at focal adhesions (Brady-Kalnay and Tonks, 1995), which are important for cell-extracellular matrix interactions and adhesion-mediated signal transduction (Wozniak et al., 2004). PTPs can either promote or suppress tumor progression and metastasis via either enhancing or suppressing cell surface receptor tyrosine kinase signaling (Sastry and Elferink, 2011). Upregulation of different forms of PTPs have been identified on the surface of the metastatic cells in other studies (Conn et al., 2008; Roesli et al., 2009). Interestingly, PTPRF has been shown to regulate adhesion between Drosophila male germ line stem cells and the niche by promoting E-cadherin-based adhesion (Srinivasan et al., 2012). It's possible role in promoting metastasis via adhesive contacts at the secondary site or pre-metastatic niche warrants further studies.

When analyzing the protein interactions in the candidate pathway we observed that three of the identified proteins, ITGA6, prostaglandin receptor negative regulator (PTGFRN) and immunoglobulin superfamily member 8 (IGSF8) interacted with two proteins (CD81 and TSPAN4) in the Moksiskaan pathway (Supplemental information S2, metastasis associated proteins p. 16 in publication I). Interestingly, CD81 and TSPAN4 are members of the tetraspanin family, a class of 33 proteins, which all have four transmembrane domains with short intracytosolic N- and C-terminal regions, and two

extracellular loops. Tetraspanins form specialized membrane microdomains on the cell surface, which control cell proliferation and migration through various adhesion and growth factor receptors. Tetraspanins interact with many different molecules, like integrins, and the composition of the complex, known as the tetraspanin web, is altered by various biological stimuli. Therefore, the different combinations of tetraspanins and associated proteins are biologically more important than individual components present at the cell surface. Recent extensive research has shown that expression of various tetraspanins and their associated partners is deregulated in human malignancies (Lazo, 2007). In regards to the PTGFRN and IGSF8, which were upregulated at the surface of the metastatic cells, it would be valuable to analyze the composition of the tetraspanin complex instead of the single proteins to better understand their role in the regulation of metastatic process.

5.2 Nucleophosmin (NPM/B23) has multiple divergent roles in breast cancer (II, III)

5.2.1 Discovery and characterization of novel NPM splice variants B23.3 and B23.4 (III)

As shown in Figure 5B we discovered that the metastatic as well as the non-metastatic cells both displayed strong nucleoplasmic NPM staining. In accordance, no overall expression difference of NPM was detected (Figure 5A). However, the metastatic cells lacked NPM on their surface and on the other hand contained cytoplasmic NPM (Figure 4A and B, respectively). Taken together these results indicated that a change in NPM localization might play a role in metastasis (Karhemo et al, unpublished data). NPM mutations are the most frequent genetic abnormalities found in acute myeloid leukemias and cause a cytoplasmic delocalization of the mutant NPM protein (Falini et al., 2005). To investigate the reason for the differential localization of NPM in the metastatic cells we cloned the gene from both metastatic and non-metastatic cells and analyzed its sequence.

Although no mutations in the sequences were found the sequence analysis revealed a possible explanation for the localization differences; the presence of two novel NPM splice variants in these cells. The domain structure and sequence alignment of the novel variants are shown in Figure 1 in publication III. One of the variants, hereafter referred to as B23.3 has recently been described in the human EST database (GenBank accession number: NM_199185). The other variant, hereafter referred to as B23.4, has not been described in the human EST database.

Characterization of the cellular localization and dimerization of the novel NPM splice variants, B23.3 and B23.4 in U2OS cells, revealed that the B23.4 could be responsible for the cytoplasmic NPM observed in the metastatic cells from which it was originally identified (Figure 4G and I in publication III). According to the immunoprecipitation analysis, B23.4 did not oligomerize with the endogenous NPM (Figure 2 in III). This is not surprising taken into account that B23.4 lacked part of the oligomerization site at the N-terminus of the NPM-protein (Figure 1 in III).

B23.3 localized to the nucleoli and nucleoplasm (Figure 4D and F and 5D and F in publication III) and oligomerized with the endogenous NPM (Figure 2 in publication III). Interestingly, different NPM forms were detected in cells overexpressing the full-length NPM (B23.1) or B23.3 indicating that they formed distinct complexes in the cell (Figure 2 in publication III). The nature of these complexes is currently unknown but the observation is important because the cellular function of NPM is heavily affected by its modifications together with homo- and hetero-oligomers. Overexpression of neither of the two novel splice variants affected proliferation of U2OS cells (Figure 3A in publication III) but both affected their phenotype and post-confluent growth (Figure 3B in III). The observed phenotypic differences were also reflected in the actin filaments visualized by phalloidin staining (Figure 3C in III).

Our results obtained from the initial characterization of the novel NPM splice variants indicated that these variants are functional and therefore most likely expressed also at protein level in the cells. NPM has been shown to play diverse cellular roles depending on the cell type. Therefore, to better

understand the biological significance of these novel splice variants it will be important to study them in other cell types in addition to the U2OS cells.

Our discovery of novel NPM splice variants in addition to the previously characterized B23.2, which lacks 35 amino acids at the C-terminus (Wang et al., 1993) adds another layer of complexity to NPM's biology and highlights the need to interpret the results obtained from NPM studies in respect to not just the localization or oligomerization but in regards to the isoforms. All the isoforms seem to either lack highly specific functional domains (B23.2 and B23.3) or only contain certain domains (B23.4) indicating that the various and often seemingly antagonistic functions of NPM might regulated by its functional domains. To shed more light on the biological significance of the novel NPM splice variants their expression pattern in normal tissues under various developmental stages and in cancer tissues of multiple origin should be analyzed.

5.2.2 Analysis of the prognostic significance of different NPM forms in breast cancer (II, III)

One approach to validate the relevance of observations made in experimental models is to analyze patient-derived material by using TMAs. Furthermore, such studies may identify candidate biomarkers for functional investigation. Despite the vast literature on NPM, to our knowledge, no analysis of the relevance of its expression or localization in regard to breast cancer prognosis was performed. Zhu et al. (2006) analyzed the nuclear expression of NPM in 44 breast cancer patient revealing that in 95% of cases (42/44) >50% of tumor cell nuclei were positive for NPM. However, the differences in intensity or prognostic value were not analyzed (Zhu et al., 2006).

To reveal the significance of NPM localization and/or expression level in breast cancer we investigated its expression level and localization in a large array of patient material (n =1160), the FinProg breast cancer database (Joensuu et al., 2003). To reveal the possible role of B23.3 , which lacked an important Thr199 phosphorylation site, in breast cancer we analyzed expression of the Thr199 phosphorylation (pThr199) in the same FinProg

database. To obtain a more comprehensive view a second breast cancer database, the FinHer cohort (Joensuu et al., 2009; Joensuu et al., 2006), was also analyzed for pThr199 (n=857). Both databases contain detailed clinical information and follow-up data for the patients enabling survival analysis and correlation with other known prognostic markers for NPM and its Thr199 phosphorylation. We also analyzed 14 histologically normal breast tissue samples originating from breast reduction surgery for their NPM expression for the comparison of NPM expression in breast cancer. Results from these studies are summarized in table 4. and discussed below.

Table 4. Different NPM forms analyzed in this study

NPM form	Observed in	Prognostic factor	Independent prognostic factor (subtype)	More frequent in	Study
Cell surface	NM-435	ND	ND	ND	
Cyto-plasmic	M-435, BCPM	No	ND	ND	I
B23.3	435	ND	ND	ND	III
B23.4	M-435	ND	ND	ND	III
High NPM levels	BCPM	Associate with with FP	Yes (luminal A)	No	II
Granular NPM	BCPM	Associate with PP	Yes (whole material, adjuvant chemotherapy)	Basal-like, unclassified	II
pThr199	BCPM	Associate with PP	No	Basal-like	III

NM-435 = non-metastatic MDA-MB-435 subclone,
M-435 = metastatic MDA-MB-435 subclone,
BCPM = breast cancer patient material,
ND = not determined, FP = favourable protgnosis, PP = poor prognosis

5.2.3 NPM expression levels affected its localization (II)

In our analysis, NPM was expressed at high levels in the luminal epithelial cells of all histologically normal breast tissue samples (Figure 1A, B and E in publication II). Similar intense, nuclear staining of the epithelial cells of the milk ducts has been detected in another study (Rower et al., 2011) indicating that under normal conditions, the luminal epithelial cells have high levels of total NPM. Interestingly, in our histologically normal breast tissue samples the younger women displayed uniform nuclear NPM localization (Figure 1A in publication II) while the older ones showed only nucleolar localization (Figure 1B in publication II). By using qPCR analysis and ectopic overexpression of NPM we showed that NPM expression level affected its localization (nucleolar or nucleoplasmic, Figure 1G-I in publication II). We confirmed the previously published results (Cicatiello et al., 2004; Skaar et al., 1998) that NPM expression is up-regulated by estrogen treatment in breast cancer cell lines (Figure 2A and B in publication) and showed that this upregulation leads to increase in the nucleoplasmic NPM (Figure 2C-F in publication II). Since estrogen levels commonly decrease at menopause in older women our results suggest that NPM expression level affected its localization in the. In accordance with our findings, UV treatment (stress) induces up-regulation of NPM expression and changes its localization from nucleolar to even nuclear distribution (Wu and Yung, 2002). If and how the effects of the stress-induced nucleoplasmic localization of NPM (Chan et al., 1985; Yang et al., 2002; Kurki et al., 2004) differ from the nucleoplasmic localization in the breast epithelial cells is unknown.

In the breast cancer samples analyzed, NPM was detected at various localizations: solely in the nucleolus (14%, Figure 3A, F in II), evenly distributed throughout the nucleus (55%, Figure 3 B, G in II), and only in the nucleoplasm (31%, Figure 3 C, H in II). We also observed a rare, cytoplasmic staining for NPM (7%, Figure 3 D, I in II). However, no correlation between the patient prognosis and these NPM localizations was observed in the study. In contrast to histologically normal breast tissue, no correlation between the NPM localization and age was detected in the tumor specimens (P = 0.67)

and no mutations explaining the differences were found (Supplemental table S1 in publication II).

5.2.4 Tumor suppressive role for NPM in breast cancer (II)

Importantly, NPM expression was reduced in breast cancer as compared to histologically normal breast tissue and patients with reduced levels of NPM showed increased risk of developing a metastatic disease as judged by distant disease free survival (DDFS, time from diagnosis to the occurrence of either metastases outside the region in which the tumor arose or death from breast cancer) (Figure 4A and supplemental Table S3 in publication II). In a subgroup analysis NPM levels showed significant prognostic value in ER-positive, $p53^{-/low}$ and HER-negative breast cancers. More importantly, NPM expression levels served as an independent prognostic factor for metastatic disease and/or breast cancer caused death in the luminal A subgroup of breast cancer (Figure 4B in II and Table 2 in II). The luminal A tumors in our analysis were classified as ER-and/or PR-positive and HER-negative, which explains the observed prognostic value in these subgroups. The prognostic value in $p53^{-/low}$-groups, which most likely have a wild type p53, can be explained by the knowledge that only about 12-15% of luminal A tumors harbor p53 mutations (Carey et al., 2006; Cancer Genome Atlas Network, 2012). Luminal B tumors differ from the luminal A ones in respect to HER2 expression. NPM expression showed no prognostic value in this subtype or in HER2-positive tumors indicting that HER2 overexpression overrode the prognostic significance of NPM.

Our results revealed that overexpression of NPM in the invasive MDA-MB-231 cells abrogated their growth in soft agar (Figure 5 in II) suggesting a tumor suppressive role for NPM in breast cancer. Contradictory to our results, high NPM expression has been associated with poor prognosis or recurrence in other tumor types (Kikuta et al., 2009; Tsui et al., 2008; Liu et al., 2012; Coutinho-Camillo et al., 2010). However, in the cases where NPM expression in the corresponding normal tissue was analyzed, it was markedly lower than in the cancer tissue highlighting the context dependent role for

NPM in neoplasia. Too much or too little NPM could disrupt the cellular homeostasis leading to deregulated growth.

5.2.5 Granular staining pattern as indicator of metastatic disease (II)

Interestingly, 14% of the breast cancer samples showed a very distinct, granular staining for NPM (Figure 3E and J in publication II). A similar staining patter has previously been identified in colorectal carcinoma (Nozawa et al., 1996) but its significance has not been analyzed. In this thesis work the presence of the granular staining pattern associated with agressiwe breast cancer characters and basal subtype (Supplemental Table S2 in publication II). Moreover, patients that showed granular NPM were more prone to develop metastatic disease or die of breast cancer than patients with non-granular NPM (Figure 4C in publication II) in several subgroups (Supplemental Table S4 in II) including luminal A (Figure 4D in II). More importantly, in a Cox proportional hazards model the granular staining pattern proved as an independent prognostic factor of distant, metastatic disease or breast cancer death in the whole series (Table 3 in II, P = 0.04). Patients displaying granular staining pattern of NPM appeared less likely to benefit from chemotherapy (Figure 4, E and F in II and Table 4 in publication II). The cause of the granular NPM is currently unknown but the mechanisms behind it would be interesting to resolve since they might shed light on the contradiction between the oncogenic and tumor suppressive functions of NPM.

5.2.6 Thr199 phosphorylation of NPM as an indicator of recurrence (III)

Interestingly, Thr199 phosphorylation (pThr199) of NPM associated with development of distant, metastatic disease and death from breast cancer (DDFS, P=0,016, Figure 7A in III) in the NPM positive (moderate or high expression) breast cancers in the FinProg dataset. This result suggest that Thr199 phosphorylation of NPM overrides the tumor suppressive effect

observed for the high total NPM levels and that NPMpThr199 represents an oncogenic form of the NPM protein. In support of this, pThr199 significantly associated with poor overall survival (*P*=0,013, Figure 7B in publication II) and distant disease recurrence, in other words metastatic disease (*P*=0,008, Figure 7C in publication II) in the FinHer material that contains patients with more aggressive disease. In this respect, the NPMpThr199 resembled the granular staining pattern. Both NPM forms were also more frequently observed in basal type breast cancers. These two forms of NPM showed inverse correlation in the FinProg breast cancer patients (Table 1 in III) indicating a connection between them. The nature of this connection, however, is totally unclear. Since nothing is known about the molecular basis of granular NPM, it is impossible to speculate the underlining mechanisms behind the similar behavior and connection between the granular NPM and Thr199 phosphorylation of NPM.

5.2.7 Thr199 phosphorylation of NPM associated with aggressive breast cancers and CIP2A expression (III)

Cancerous inhibitor of protein phosphatase 2 (CIP2A) has recently been identified as an oncogene (Junttila et al., 2007). Intriguingly, we demonstrated that NPMp Thr199 associated with CIP2A expression in both breast cancer cohorts analyzed (Table 1 in III and Figure 6A in publication III). Moreover, a significant reduction in pThr199 levels was observed at day 4 and 5 after siRNA mediated silencing of CIP2A (Figure 6B in III) confirming the association observed in the patient analysis. CIP2A also seemed to associate with total NPM levels in the patient material. In accordance, a slight increase in total NPM levels was evident at day 3 after CIP2A silencing, but this was not detected at later time points (Figure 6B in III).

Thr199 of NPM is dephosphorylated by a protease distinct from PP2A, PP1beta (Lin et al., 2010). In addition, CIP2A silencing increased the pThr199 at day 3 after which a clear reduction was observed (Figure 6B in

III). This together with the different cellular localizations of CIP2A and NPM indicate that Thr199 is not a direct target of PP2A.

The expression of CIP2A (Côme et al., 2009; Niemela et al., 2012) and the NPMpThr199 both associated with aggressive characteristics of breast cancer (p53 expression indicative of mutant p53 and basal-type, Table 1 in publication III). These two proteins participate in the regulation of the same pathways like c-myc (Niemela et al., 2012; Bockelman et al., 2012; Guo et al., 2000; Neiman et al., 2001; Kim et al., 2000; Zeller et al., 2001; Li and Hann, 2013; Li et al., 2008) and E2F1 (Lin et al., 2010; Lin et al., 2006; Takemura et al., 2002; Takemura et al., 1999; Liu et al., 2009; Laine et al., 2013). In addition, in our analysis, pThr199 significantly associated with EGFR expression (Table 1 in publication III) and the CIP2A has shown to be regulated by the EGFR-MEK1/2 ETS signaling pathway (Khanna et al., 2011). The detailed molecular mechanism behind the connection between NPMpThr199 and CIP2A warrants further experimental analyses to resolve if CIP2A is responsible for the association between NPMpThr199 and development of metastatic disease in breast cancer.

5.2.8 NPM modifications and metastasis?

In this thesis, by using two distinct large breast cancer TMA datasets, we have revealed that, in respect of development of metastatic disease and breast cancer death, different NMP forms have contradictory roles in breast cancer (summarized in Table 4). To our knowledge, this is the first analysis comparing the significance of different NPM forms (total levels, granular NPM and Thr199 phosphorylation) in any cancer material. Interestingly, high total NPM levels associated with favourable prognosis and reduced risk of metastatic disease and/or death from breast cancer only in luminal A breast tumors while its expression did not significantly vary among the different subtypes (Supplemental Table S2 in publication II). On the contrary, granular staining and Thr199 phosphorylation were more frequent in basal-like breast cancers and served as indicators of metastatic disease in whole patient material. As described in the Review of the literature, NPM

participates in the regulation of p53 and RB-pathways. Interestingly, vast majority of luminal A tumors harbor wt p53 while this protein is mutated or inactivated in most BLBCs (Carey et al., 2006; Cancer Genome Atlas Network, 2012; Dumay et al., 2013). In addition, in most BLBCs tumor suppressor RB is affected (Gauthier et al., 2007; Herschkowitz et al., 2008; Subhawong et al., 2009). If these molecular differences affect the role of NPM and its different forms in breast cancer should be analyzed in the future.

Previously, in a comparative proteomic analysis the phosphorylation of NPM serine 125 has been shown to be more abundant in cancer samples than in controls (Rower et al., 2011). In addition, a 2D gel analysis identified an unknown NPM modification to be more abundant in metastatic breast tumors than in their non-metastatic counterparts (Vydra et al., 2008). Moreover, this thesis works revealed a novel NPM splice variant from metastatic cells and a connection between NPMpThr199 and a known oncogene and modulator of post-translational modifications, CIP2A. Taken together these findings propose that postranslational modifications and domain structure changes affecting these modifications in NPM might be general mechanisms to control NPM's function in breast cancer. To make the picture more complicated, NPM's oncogenic potential is also modified by plakoglobin (PG, γ-catenin), a homolog of β-catenin with dual adhesive and signaling functions (Lam et al., 2012) and HLJ1, a tumor suppressor and a member of the heat shock protein 40 chaperone family (Chang et al., 2010). In addition, NPM has been shown to suppress of CXCR4-mediated G protein activation and chemotaxis via directly interacting with CXCR4. CXCR4 is the primary receptor for CXCL12, which is secreted by metastatic sites and promotes homing of CXCR4 expressing tumor cells to these sites (Zhang et al., 2007).

6 CONCLUSIONS AND FUTURE PROSPECTS

One aim of this thesis work was to identify novel cell surface proteins associated with metastasis colonization and dormancy. Importantly, this study resulted in the discovery of a several novel metastasis associated proteins and opened up interesting connections for further studies. Out of the identified proteins, CD109 proved as a promising novel candidate for future analyses. In addition to the conventional cell surface proteins the proteomic comparison revealed novel cell surface proteins. Intracellular proteins are increasingly observed in extracellular space highlighting the need to reconsider the classical protein localizations. Importantly, in respect to drug target discovery a protein that is a therapeutic target in one cellular location may have anti-target activity elsewhere.

The present study demonstrated for the first time analysis of different NPM forms in the same patient material. Strikingly, different NPM forms (total levels, granular staining and pThr199) behaved in opposite ways indicating the need to critically evaluate results concerning NPM's function and prognostic relevance. NPM cannot be considered as a single entity but in the future more effort should be put to analyses to study if similar relationships are present in other tumor types. In respect to NPM as a drug target one should carefully consider all the possible effects the drug might have on NPM's function.

The pThr199 associated with CIP2A expression. The positive prognostic role of NPM was evident in luminal A tumors, which rarely harbor p53 mutations. The poor prognostic function was evident in basal type breast cancer commonly harboring a mutant p53. The connection of NPM to p53 and CIP2A should be analyzed at functional level.

Finally, this study revealed the presence of two novels splice variants of NPM, B23.3 and B23.4, in cancer cells. In the future their molecular function, expression pattern at the tissue level and possible role in regulation of metastatic dissemination of cancer should be studied.

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